Master's thesis
Degree Programme in Materials Research
Polymer Materials Chemistry

ENZYMATIC TREATMENT OF SOFTWOOD KRAFT PULP AT HIGH AND LOW CONSISTENCY

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17.08.2020

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UNIVERSITY OF HELSINKI
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MATEMAATTIS-LUONNONTIETEELLINEN TIEDEKUNTA
MATEMATISK-NATURVETENSKAPLIGA FAKULTETEN
FACULTY OF SCIENCE

Tiedekunta – Fakultet – Faculty	Koulutusohjelma – Utbildningsprogram – Degree programme
Faculty of Science	Master of Science

Opintosuunta – Studieinrikting – Study track

Materials Research Master's programme, Polymer Materials Chemistry

Tekijä – Författare – Author

Elisa Jasmin Spönla

Työn nimi – Arbetets titel – Title

Enzymatic treatment of softwood kraft pulp at high and low consistency

Työn laji – Arbetets art – Level	Aika – Datum – Month and year	Sivumäärä – Sidoantal – Number of pages
Master's Thesis	08/2020	69

Tiivistelmä – Referat – Abstract

The aim of the thesis was to study enzymatic treatment as a way to modify paper grade pulp to be a suitable raw material for the future textile industry. Wood as a raw material is an environmentally friendly option for textile production but its sustainable exploitation is not easy. Currently, ionic liquids are assumed to enable a safe and sustainable process for the production of wood-based regenerated fibres. These processes commonly use dissolving pulp as their raw material but replacing dissolving pulp with a paper grade kraft pulp would decrease environmental impact and production expenses. In this work, molar mass distribution of softwood paper grade kraft pulp was selectively modified using enzymes. Enzymes were utilized instead of acids because of their favourable abilities to selectively modify targeted polymers and to increase fibre porosity.

Enzymatic modifications of softwood kraft pulp were performed to decrease degree of polymerization of cellulose and lower the quantity of hemicellulose. Hydrolysis of cellulose was catalysed with endo-1,4-β-glucanase (endoglucanase) and hemicellulose was degraded using endo-1,4-β-mannanase and endo-1,4-β-xylanase. The treatments were carried out both at high (20%) and low (3%) pulp consistency to examine the synergistic effect of enzymatic and mechanical action arising in the high consistency treatment. Additionally, influence of different enzyme combinations on the pulp properties was studied. The modified pulp samples were characterized by determining intrinsic viscosity, molar mass distribution, yield loss, and its composition. The fibres were imaged with light microscopy.

The degree of polymerization of the pulp cellulose was successfully decreased with a relatively small endoglucanase dose. The amount of hemicellulose was reduced by removing 11% of the total galactoglucomannan and 40% of the total arabinoglucuronoxylan. The high consistency treatments decreased intrinsic viscosity 1.9 times more on average than the low consistency treatments. The high consistency treatments were effective with low enzyme doses, easy to control, and reliably repeated. Therefore, enzymatic pulp treatment at high consistency seems to be a compatible way to modify paper grade kraft pulp to suitable raw material for textile production. Further studies related to pulp dissolution in ionic liquids, fibre spinning, and fibre regeneration should be concluded to confirm applicability of the modified fibres.

Avainsanat – Nyckelord – Keywords

cellulose, enzymatic treatment, endoglucanase, hemicellulase

Säilytyspaikka – Förvaringställe – Where deposited

Helda / E-thesis

Muita tietoja – Övriga uppgifter – Additional information

Preface

The Master's thesis was performed at VTT Technical Research Center of Finland as a part of GRETE project in Espoo. This thesis is a part of a project that has received funding from the Bio Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation programme under grant agreement No 837527.

I greatly enjoyed working at VTT because of the welcoming atmosphere and skilled personnel. I would like to express my biggest gratitude to my supervisors Jenni Rahikainen, Matti Siika-Aho, and Stina Grönqvist for giving me the opportunity to participate in this interesting project. I am grateful for their time, continuous encouraging guidance, and the many inspiring and helpful discussions on theoretical and experimental topics.

I would like to thank professor Sirkka-Liisa Maunu for her supportive feedback and contribution. Additionally, I am thankful to Mariitta Svanberg and Riitta Alander for sharing their profound knowledge of laboratory practises and the practical utilization of enzymes. I would like to acknowledge Jenni Limnell for her help in sample preparation for size exclusion chromatography. I want to thank Atte Mikkelson for his expertise in operating size exclusion chromatography and high performance liquid chromatography and Nina Vihersola and Juha Haakana for performing the viscosity analysis. I would like to acknowledge Atte Mikkelson and Tiina Liitiä for discussing size exclusion chromatography of pulp samples and for the help in interpreting the size exclusion chromatography results.

I would like to thank Jenna, Eveliina, Pinja, Juulia, and Lauri for peer support and interesting discussions during many lunch breaks. Finally, I am grateful to Adrien and my family for their love and support they gave me during this work.

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List of abbreviations

AGU	anhydroglucose unit
CED	copper ethylenediamine
CMC	carboxymethyl cellulose
CS_2	carbon disulphide
Da	Dalton
[DBNH][OAc]	1,5-diaza-bicyclo[4.3.0]non-5-enium acetate
DMAc	*N,N*-dimethylacetamide
DMI	1,3-dimethyl-2-imidatzolidinone
DNS	dinitrosalicylic acid
DP	degree of polymerization
DS	degree of substitution
EIC	ethyl isocyanate
HCl	hydrogen chloride
Hi	*Humicola insolens*
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
IL	ionic liquid
IUPAC	International Union of Pure and Applied Chemistry
kat	katal, mol/s
LiCl	lithium chloride
M	mol/l
M_n	number average molar mass
M_w	weight average molar mass
MALS	multiangle laser light scattering

NaOH	sodium hydroxide
NMMO	*N*-methylmorpholine-*N*-oxide
NMR	nuclear magnetic resonance
PD	polydispersity index, M_w/M_n
RI	refractive index
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
Tr	*Tricoderma reesei*
wt%	weight percent
w/v	weight by volume

INTRODUCTION

World's population continues to grow and is estimated to increase by 2 billion during the next 30 years according to the United Nations.[1] Population growth combined with the improving standard of living in many developing countries strongly increases the need of food and doubles the demand of raw materials by 2060.[2,3] In 2017, 103 million tons of fibres for textiles was produced globally and this was 3.7 percent more than the previous year continuing the fast growth of the textile industry.[4]

Most of these produced fibres were oil-based synthetic man-made fibres comprising almost 63 percent of the total production.[4] Annually, the washing of oil-based clothes is estimated to discharge half a million tons of microplastic fibres to the ocean.[5] The problem of microplastics and the limited fossil resources are demanding to pay attention to other textile materials and how their production could be scaled up for the growing need. In 2017, 25% of the global production consisted of cotton, under 6% of other natural fibres like wool, and over 6% of wood-based manmade cellulosic fibers.[4] Natural fibres have several advantages over the oil-based synthetic fibres. They are comfortable to wear due to their breathable and moisture absorbing structure and most importantly they are biodegradable and made of renewable materials.[6]

Cultivation of the major natural fibre source, cotton, is problematic because of the huge amount of land, water, fertilizers, and pesticides it requires.[7] Additionally, cotton cultivation is competing with food production which will be a major concern in the future when more of the fertile agricultural land will be needed to produce food for the growing population.

Currently, several wood-based textile fibres like viscose and lyocell are being produced industrially. Wood as a raw material is renewable, biodegradable, and doesn't compete with food production as cotton cultivation. Besides, the production of cotton needs a larger area and more water than the production of wood-based fibres. The production of lyocell fibres requires 0.24 hectares of land to produce one ton of fibres annually while viscose, irrigated cotton, and rain-fed cotton require 0.69, 1.00, and 2.35 hectares respectively.[8] The amount of water needed to produce one kilogram of lyocell or viscose is 265 and 445 litres respectively while the production of genetically modified cotton and conventional cotton requires 6500 and 8800 litres.[8]

In 2017, viscose was dominating the market of man-made cellulosic fibres with an 85% share of the annual production.[9] Despite the great properties of viscose, its production process poses severe risks to people and the environment. The manufacturing procedure utilizes toxic carbon disulphide (CS_2) in a temporary derivatisation step to form soluble cellulose xanthate.[10] The method is commonly used even if the serious occupational and environmental risks of CS_2 are well known.[11–13]

The Lyocell process is a newer and less harmful method to produce wood-based textiles.[14] The process utilizes *N*-methyl morpholine-*N*-oxide monohydrate (NMMO) where the cellulose fibres are dissolved without derivatisation. Recovery of NMMO and the cellulose yield are high but the solvent has thermal and chemical stability issues.[14] Another downside of NMMO is the high viscosity of the spinning dope already at moderate cellulose concentrations which weakens the strength properties of the generated fibres and limits the process economy.[15]

Promising novel cellulose solvents with higher chemical and thermal stabilities have been discovered from the class of ionic liquids (IL).[15–17] ILs are a large group of diverse salts that have low melting temperatures.[18] Due to their promising features, ILs are assumed to enable a safe and sustainable process for the production of wood-based textiles and later replace the use of NMMO.[15,19,20] One example of these potential solvents is 1,5-diaza-bicyclo[4.3.0]non-5-enium acetate ([DBNH][OAc]) which is used in a process similar to lyocell manufacturing and is called Ioncell-F.[15,19] [DBNH][OAc] is a powerful cellulose solvent and viscosity of the resulting cellulose solution is lower than in the process with NMMO which allows preparation of high concentration dopes and processing at milder conditions. Lower processing temperature degrades cellulose less which decreases yield loss and improves the strength properties of the regenerated fibres.[15]

Regenerated cellulosic fibres are mostly produced from dissolving pulp which has higher cellulose and lower hemicellulose and lignin content than paper grade kraft pulp.[21] Kraft pulping is the most utilized pulping method in the world and it can be utilized to produce dissolving grade pulp with an additional prehydrolysis treatment before the actual pulping.[22] The additional prehydrolysis enhances the removal of hemicellulose and decreases the degree of polymerization (DP) of cellulose.[22] A high content of shorter hemicellulose chains decreases the quality of the regenerated fibres and leads to enriching of the short chains in the spinning solution which complicates the recycling of the solvent.[10,23] DP of cellulose is decreased to have a suitable viscosity for fibre spinning.[22]

The use of paper grade pulps in the textile industry would lower the environmental impact and decrease production expenses.[24] Paper grade kraft pulp may be altered to be a suitable raw material in the textile production by decreasing the DP of cellulose and reducing the amount of hemicellulose. In this work, the DP of cellulose and the content of hemicellulose of softwood paper grade kraft pulp was modified by enzymatic treatment at high and low pulp consistencies. The utilization of enzymes over acid treatment was selected for the selective hydrolysis of targeted polymers and for the ability to increase fibre porosity.[25,26] Increased porosity is a desirable feature since it facilitates the dissolution of fibres in ionic liquids or other solvents.[27]

THEORY

1 Wood fibres

1.1 Structure of wood fibres

Natural wood consists mainly of polymeric structural components such as cellulose, hemicellulose, and lignin. Cellulose is a homopolysaccharide consisting of linearly linked β-D-glucopyranose units.[28] Hemicellulose is a heterogeneous group of short polysaccharides with a linear backbone and various side chains.[29] Hemicelluloses are amorphous heteropolymers with several monomeric sugar units. Lignin is a heterogeneous copolymer consisting of different phenylpropanes as its structural units.[29] This cross-linked phenolic compound is polymerised of sinapyl, p-coumaryl and coniferyl alchohol precursors. Cellulose content is about 40– 45% of the wood dry solid weight in both soft- and hardwoods but the ratio of hemicellulose and lignin differs.[29] Softwoods contain 25– 30% of hemicellulose and lignin while hardwoods have a higher content of 30-35% of hemicellulose and a lower content of 20–25% of lignin.[29] In smaller quantities, wood contains other polymeric substances like starch and pectin and some non-structural compounds such as low molar mass extractives, water-soluble organics, and inorganics.[29]

Wood consists of several types of cells with diverse functions, orientation, structures, and chemical compositions and they vary between wood species.[29] The vertical and long cells whose main function is to give mechanical strength to the three and also transport liquid in softwoods are referred to as wood fibres. Most of the cellulose in wood is found from these fibres. Typical fibres are 1.4–6 mm long in most of the softwoods and 0.4–1.6 mm long in most of the hardwoods.[29] These cells are bound together by the high lignin content of the middle lamellae around them.[28] The fibres have thick cell walls consisting of two main layers. The layers differ from each other by their structure and chemical composition.[28] The outermost layer of the wood cell wall is the thin primary wall (P). Its major component is lignin which encloses all the other minor components such as cellulose and hemicellulose.[29] Underneath the primary wall is the second main layer called the secondary cell wall which mainly composes of cellulose but contains also some hemicellulose and lignin.[29] The secondary cell wall consists of three distinct sublayers that have different thicknesses and orientation of cellulose microfibrils. Outer (S_1) and inner (S_3) layers of the secondary cell wall are thinner and have a larger angle to the cell axis whereas the middle layer (S_2) of the secondary cell wall is thick and its cellulose microfibrils are more aligned with the cell axis.[21] Additionally, some wood species have a thin membrane called warty layer inside the inner layer of the secondary cell wall.[28] The wood cell wall layers are illustrated in Figure 1.

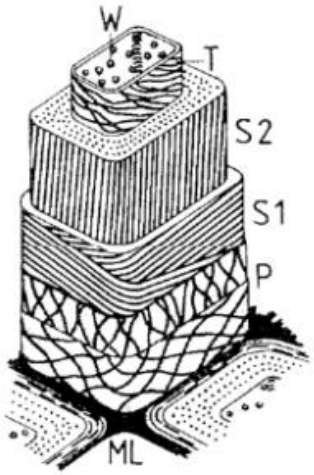


Figure 1. Schematic drawing presenting wood cell wall layers and microfibril orientation. The abbreviations are ML middle lamella, P primary wall, S1 outer layer of the secondary cell wall, S2 middle layer of the secondary wall, T inner layer of the secondary wall (tertiary wall), and W warty layer. Figure reused with permission from Pedro Fardim, 2011, Chemical Pulping Part 1, Fiber Chemistry and Technology, Paper Engineers' Association.[30]

The smallest independent structural unit of a wood cell wall is a cellulose microfibril.[31] Cellulose microfibrils compose of parallel cellulose chains held together by hydrogen bonds.[21,31] and their diameter is between 1.5 and 3.5 nm.[32] Cellulose microfibrils are long but thin, have crystalline and amorphous areas and anisotropic physical properties[31] Cellulose microfibrils form larger microfibrils with a diameter of 10–30 nm and length of several hundred nanometers hold together by surrounding hemicellulose and lignin.[32] These larger microfibrils are organized parallel to each other forming even larger structures like lamellas or microfibril bundles while hemicellulose and lignin exist between them (Figure 2).[21,32] Hemicellulose is oriented with cellulose microfibrils and has hydrogen bonds with cellulose chains[33] whereas lignin is isotropic and has covalent linkages mainly with hemicellulose but also with cellulose.[34]

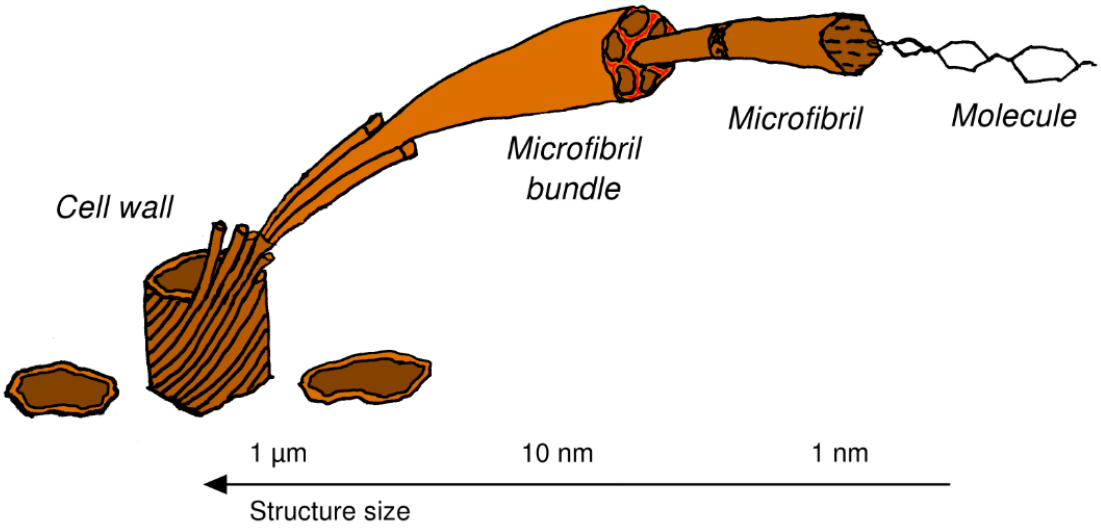


Figure 2. Hierarchical structure of the wood cell wall and microfibril bundles. Figure reused with permission from "Penttilä, P., 2013, *Structural characterization of cellulosic materials using X-ray and neutron scattering*, University of Helsinki".[35]

1.2 Cellulose

1.2.1 Chemical structure

Cellulose is a homopolysaccharide consisting of β-D-glucopyranose units which are linearly linked together by (1→4) glycosidic bonds.[21] This means that the repeating anhydroglucose units (AGU) are connected between the equatorially oriented hydroxyl group of the C1 carbon and the C4 carbon of the next unit. Every second AGU is rotated 180° to enable better angles for the glycosidic bonds.[32] The disaccharide of two joined AGUs is called cellobiose.[36] Structure of cellulose is illustrated in Figure 3.

Cellulose is the most abundant polymer on Earth.[32] It has high tensile strength and therefore it appears mostly as a structural component in the cell walls of plants but also in some certain bacteria and algae.[37] The content and the degree of polymerization (DP) are strongly dependent on the origin of the cellulose.[32] Natural wood is the most important commercial source of cellulose fibers as it contains 40–45% of cellulose as the dry weight.[29] Cellulose is a polydisperse material but the degree of polymerization in natural wood is about 10 000 while DP of cotton cellulose is usually 15 000.[28] However, DP can decrease to 500–2000 as a result of chemical pulping.[28]

The high tensile strength of cellulose is caused by the many intra- and intermolecular hydrogen bonds that are responsible for the occurrence of highly crystalline areas among the amorphous regions.[32] The hydrogen bonds affect the general structure of cellulose, its reactivity, and chemical behaviour. The crystallinity of this semicrystalline polymer varies between the cellulose sources and could be over 80% in algae but lower in plants.[32] The hydrogen bonds within the cellulose chain and between the

adjacent chains exist because of the hydroxyl groups in the repeating units.[32] Each anhydroglucose unit contains three equatorially oriented hydroxyl groups which two of them are secondary alcohols and one is a primary alcohol.[21]

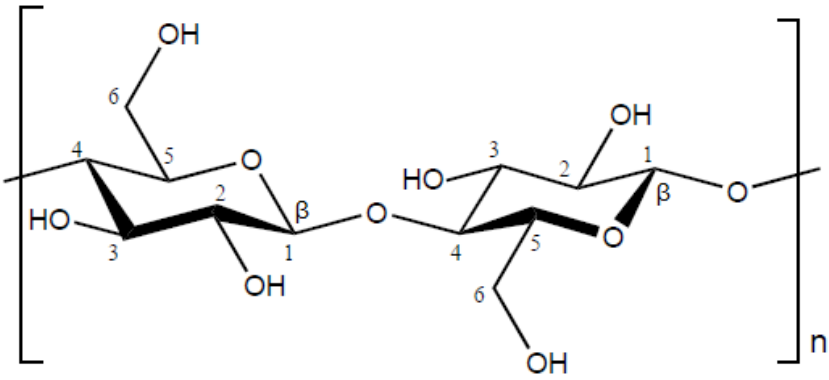


Figure 3. Structure of cellulose showing two D-glucopyranose units linked by β(1→4) glycosidic bond.

1.2.2 Crystal structure

As a semicrystalline material, cellulose contains both highly ordered and amorphous areas. Crystalline regions of cellulose are less accessible making them less reactive.[32,38] These highly ordered areas can exist in different allomorphs where the intra- and intermolecular hydrogen bonds are located differently.[32] In nature, cellulose always occurs as cellulose I which has two subclasses: I_α and I_β.[39] These allomorphs are found beside each other even within the same microfibril.[31] Most of the cellulose in higher plants is I_β form and bacterial and algal celluloses consist mostly of I_α.[39] Chains are packed parallel in both cellulose I_α and I_β crystal structures.[40] However, cellulose I_α has triclinic unit cells composing of one chain and I_β has monoclinic unit cells composing of two chains.[41,42] Additionally, the unit cell sizes are different and the planes formed by the hydrogen bonds are displaced differently on top of each other in these allomorphs.[31,41,42] The cellulose chains form sheets that are connected with van der Waals bonds.

Other crystalline forms of cellulose can be obtained as a result of regeneration, heating, or chemical modification.[32] The modified allomorphs are called cellulose II, III, and IV. Cellulose II is thermodynamically the most favourable of all allomorphs and can be formed from cellulose I by sodium hydroxide treatment or regeneration after dissolving.[21,32] Because of improved packing energy and stronger hydrogen bond network, this transformation of allomorph is permanent and cellulose II cannot be reconverted back to cellulose I.[21,43] Besides, in the allomorph II, cellulose sheets are connected with hydrogen bonds unlike in any other allomorph. Regenerated cellulose II has

antiparallel chains and a monoclinic unit cell composing of two chains.[43] Wood-based manmade cellulosic fibres contain cellulose in allomorph II which is the reason of the silk-like texture of these fibres.[32]

Cellulose allomorphs of III and IV are created when celluloses I or II are modified with heat or chemicals. Transition to cellulose III is reversible and can be further irreversibly modified to cellulose IV. Transition to cellulose IV is reversible if it is prepared straight from cellulose I or II. Both of cellulose III and IV have two different allomorphs which directly depends whether the crystals are formed from cellulose I or cellulose II.[44]

1.3 Hemicellulose

Hemicellulose is a group of linear and amorphous heteropolysaccharides with variable side chains. They mostly compose of different hexose and pentose sugar units but also contain some deoxyhexoses and uronic acids. Most of the hemicelluloses are short and have DP of only 100–200. In addition to the varying hemicellulose content between soft- and hardwoods, also the amount of each hemicellulose constituent and their chemical compositions differ.[29]

The main hemicellulose components in wood are glucomannan and xylan. Softwood glucomannan is galactoglucomannan and it is the most common hemicellulose in softwood with 15–20% content of the total dry wood. The linear backbone of galactoglucomannan is formed of linearly (1→4) linked β-D-glucopyranose and β-D-mannopyranose units. Additionally, the main chain is substituted with (1→6) linked single α-D-galactopyranose units but the occurrence of the substituent differs (Figure 4). In the galactose rich area, the ratio between galactose, glucose, and mannose is 1:1:3 respectively whereas it is 0,1-0,2:1:3 in the galactose poor area. Moreover, every 3–4 hexose unit of the backbone is substituted by O-acetyl groups through the hydroxyl groups at carbons C2 and C3. On the other hand, hardwood glucomannan doesn't contain galactose or acetyl groups and it appears in lower quantity.[28]

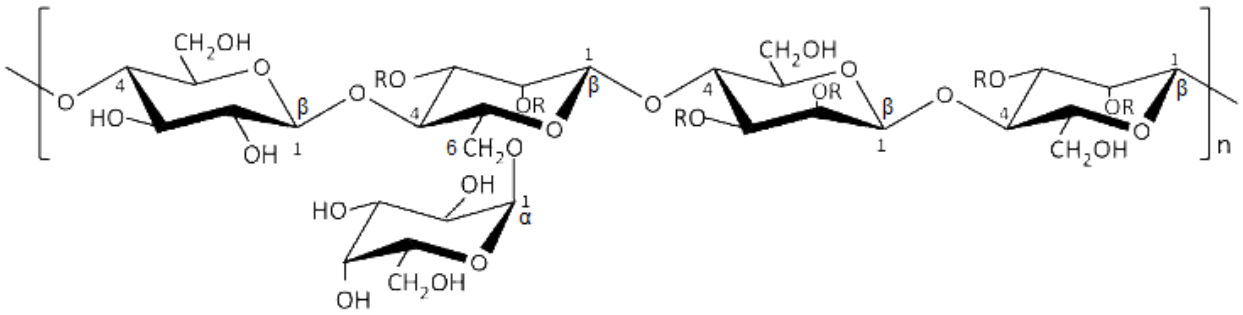


Figure 4. Structure of galactoglucomannan.

In softwood, 5–10% of the dry wood weight is xylan. Xylan in softwood is arabinoglucuronoxylan and its backbone composes of linearly (1→4) linked β-D-xylopyranose units. The chain has branches of (1→2) linked 4-O-methyl-α-D-glucuronic acid and (1→3) linked α-L-arabinofurose (Figure 5). The mass ratio of arabinose, 4-O-methyl-α-D-glucuronic acid, and xylose is 1:2:8 respectively. The softwood xylan does not contain any acetyl groups. In hardwood, glucuronoxylan is the most common hemicellulose and it consists of only 4-O-methyl-α-D-glucuronic acid and xylose. Additionally, the hardwood xylan contains less uronic acid constituents but more acetyl groups than the softwood xylan. Hardwood xylan also contains small quantities of L-rhamnose and galacturonic acid for example.[28]

Furthermore, several other types of hemicelluloses are present in smaller quantities. Few examples of these are different galactans that appear in reaction and compression wood and arabinogalactan which usually makes under 1% of softwood dry weight but can make up even 20% of hartwood of larches.[29]

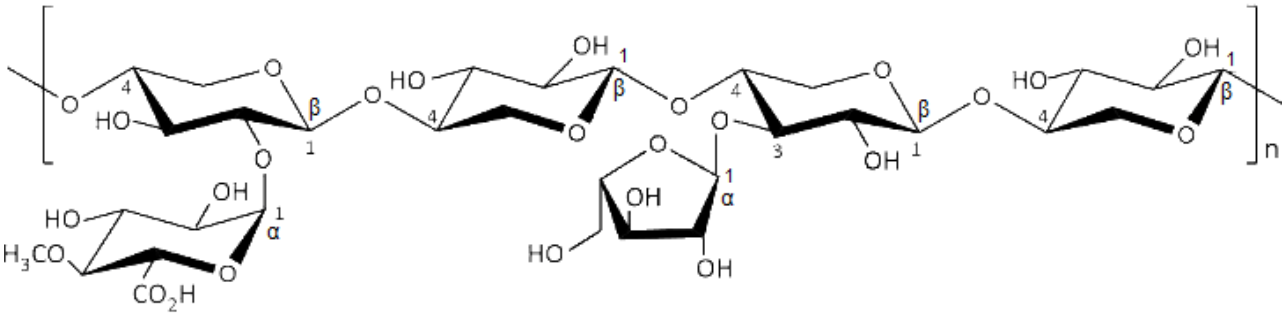


Figure 5. Structure of arabinoglucuronoxylan.

1.4 Pulping

Wood pulping is a process to separate and collect cellulose fibres from the wood cell wall.[21] In traditional pulping, the amount of hemicellulose and lignin is decreased by degrading and dissolving them while trying to prevent decomposing or losing cellulose.[21] Pulping can be mechanical, chemical, or a combination of these two methods. High yield pulps can be gained with mechanical pulping but simultaneously the lignin content remains high.[45] Better removal of lignin can be achieved with chemical delignification.[45] The two most commonly used pulping methods sulphate (kraft) and sulphite pulping, which kraft method dominates, are both chemical processes.[46] Kraft pulping is mostly used for paper production whereas sulphite pulping or kraft pulping combined with acid prehydrolysis produces dissolving pulp which is used in the manufacture of textiles and cellulose derivatives such as

cellulose triacetate and cellulose ethers.[45] In kraft pulping, the amount of lignin and hemicellulose decrease in highly alkaline conditions at high temperatures.[46] Similarly, the sulphite process is operated at high temperature but contrariwise at low pH with sulphites and bisulphites.[46] Sulphite process results in pulp with higher yield but lower strength properties than kraft pulping.[45]

The kraft process is started by steaming wood chips to remove air.[46] A warm chemical mixture called white liquor containing mainly sodium hydroxide and sodium sulphide is added on the chips to initiate delignification.[46] Lignin degrades when hydroxyl and hydrogen sulphide ions from the white liquor cleave ether linkages between its repeating units.[45] Simultaneously, the hydrophilicity of lignin increases and it dissolves better in the cooking liquor as its hydroxyl groups are liberated.[21] The cooking temperature is kept high until the desired lignin content is achieved.[45] Additionally, the high cooking temperature combined with the alkaline conditions leads to the hydrolysis of glycosidic bonds of polysaccharides like cellulose and hemicellulose.[21] Vulnerability on hydrolysis depends on the structure and accessibility of the polysaccharides. That is why cellulose with a high degree of polymerization and crystallinity rate degrades less than amorphous hemicellulose.[21]

The cooking is ended when about 90% of lignin is removed.[47] The resulting pulp is collected and washed and the cooking chemicals are recovered.[46] The pulp is bleached by removing residual lignin using NaOH and other bleaching chemicals such as oxides, chlorine dioxide, hydrogen peroxide, or ozone.[21] Alternatively, the pulp can be bleached moderately by destroying the chromophores of lignin with sodium dithionate or hydrogen peroxide.[18] After the bleaching, the yield of kraft pulp is 43–45%.[45]

2 Enzymes

Enzymes are a large group of macromolecules that act as specific biocatalysts. Characteristic for enzymes is their high specificity, meaning that they typically catalyse reactions to a highly specific substrate structure. Enzymes are proteins built up from one or several chains composing of amino acid residues that are linearly linked via amide linkages in a certain sequence. Together the amino acid composition and their sequence determine the three-dimensional protein conformation which is required for the function as a catalyst.[48]

Temperature and pH have an effect to enzyme activity. As temperature increases, catalysis and hydrolysis are thermodynamically more probable which leads to an increase in reaction rate provided that the enzyme remains stable.[49] If the temperature rises too high, the active site of an enzyme can change reversibly to an inactive form, or the entire enzyme can become denatured irreversibly. Depending on the temperature, all of the enzymes present are not necessarily inactivated but there is

a temperature dependant ratio between the active and inactive forms.[49] The amount of inactive form increases with increasing temperature.[49] Decreased enzyme activity can be partly compensated by more probable thermodynamic conditions for the catalysis and reaction. Therefore temperature influences the number of catalysed reactions at a certain time. Similarly, the ratio between the active and inactive forms of an enzyme is affected by pH.[50] The optimum pH area depends on the pKa values of the acidic and basic groups in side chains of the active site.[50] An active site is the area of an enzyme where substrate molecules bind and are catalysed.

Cellulose is degraded by three types of hydrolases that are catalysing the cleavage of the glycosidic bond between the β-D-glucopyranose units. These enzymes are secreted by fungi or bacteria and they either act simultaneously or sequentially. Endoglucanases (EC 3.2.1.4) hydrolyse the chain internally while exoglucanases (cellobiohydrolases, EC 3.2.1.91) cleave from chain ends producing cellobiose.[51] Cellobiose and other small soluble oligosaccharides are further degraded to glucose by β-glucosidases (EC 3.2.1.21).[51] All enzymes have catalytic a domain but many polysaccharide degrading enzymes like cellulases have additional carbohydrate-binding modules which bind to the substrate surface.[52,53]

Endoglucanases act in a random manner but they usually have higher activity towards the amorphous areas of cellulose.[54] Based on the hydrolysis mechanisms, endoglucanases can be divided into inverting and retaining cellulases depending on the anomeric configuration after the reaction.[55] The configuration of the anomeric carbon is inverted by a one-step reaction including an oxocarbenium ion-like transition state (Figure 6) while the configuration is retained by a two-step reaction including a glycosyl-enzyme intermediate and two oxocarbenium ion-like transition states.[51]

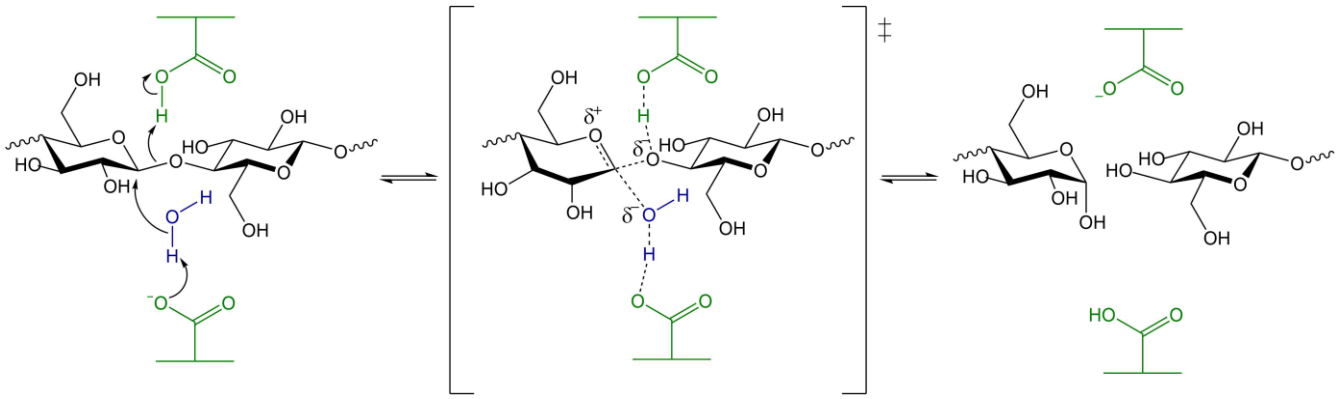


Figure 6. Mechanism of endoglucanase initiated cellulose hydrolysis by inverting mechanism.

Endoglucanases have been exploited to lower DP of pulp cellulose and thereby decrease pulp viscosity to a wanted level.[56–59] Enzymatic modification of fibres is capable to increase porosity of the fibre cell wall, unlike acid treatment.[25,26] Increased porosity is a desirable feature since it enhances the fibre accessibility and reactivity towards solvents and reagents.[27] Additionally, enzymatic treatment is shown to increase the microfibrillar distance which suggests opening of microfibrillar bundles in the cell wall and increased accessibility.[60]

Hemicellulose is degraded by glycoside hydrolases which catalyse the hydrolysis of the glycosidic bonds and carbohydrate esterases that catalyse the hydrolysis of ester linkages in the side chains. Since hemicellulose is a heteropolymer, it has various glycosidic linkages between the different sugar units, and diverse enzymes are needed for its degradation. The linkages and sugars in the back chains of the different hemicellulose polysaccharides differ and require their own enzymes. However, the similarities in the side chains allow the same enzymes to participate in the degradation of several polysaccharides.[53]

Glucomannan and galactoglucomannan backbone are degraded by β-mannanase (1,4-β-D-mannan mannohydrolase), which catalyses the hydrolysis of internal β-1,4-glycosidic bonds and by β-mannosidase, which releases mannose from the nonreducing end of β-1,4-linked mannoside, and by β-glucosidase which releases glucose from the nonreducing end.[53] Additionally, some endoglucanases like *Tricoderma reesei* Cel45A are able to hydrolyse galactoglucomannan backbone next to the glucose units.[61] The activity of β-mannanase and the selection of probable hydrolysis location on the glucomannan backbone are strongly affected by its DP and the degree and placement of the side-chain substitution.[62] Therefore, the complete degradation of glucomannan requires additional enzymes like acetyl mannan esterase and α-galactosidase to cleave the side chain substituents.[62]

Xylan and arabinoglucuronoxylan backbones are degraded by β-xylanase (1,4-β-D-xylan xylanohydrolase), which catalyses the hydrolysis of internal β-1,4-glycosidic bonds, and β-xylosidase which releases xylose from the nonreducing end. Similarly, as for β-mannanase, the DP of the backbone and the degree and placement of substitution effect on the selection of probable hydrolysis location and the β-xylanase activity. The full degradation of xylan requires also additional hemicellulases like α-glucuronidase and α-arabinofuranosidase to degrade the side chain substituents.[53]

2.1 High consistency enzymatic treatment

Previously enzymatic treatments on pulp fibres have mostly been conducted at low pulp consistency.[57,58,63] However, recently several studies have shown how combined mechanical and enzymatic treatment at high pulp consistency modifies the pulp more effectively than the low consistency treatment.[56,64–66] At high consistency, mechanical and enzymatic treatments have been discovered to work synergistically and therefore more efficiently simultaneously than sequential.[64,65] At high consistency the enzymatic action is enhanced by two ways, by higher enzymatic adsorption, and by simultaneous mechanical action.

At high consistency, the enzymes have been proved to adsorb more on the substrate surface.[65,67] This increases the probability of the formation of the enzyme-substrate complex and catalysis.[67] Mixing at high consistency generates friction between the fibres which causes delamination of the S2 layer of the fibre cell wall.[56] The delamination is generated by mechanical mixing but is further increased by the enzymatic hydrolysis. Rahikainen et al. found mixing at high consistency (20%) alone to increase total and micropore volumes of the fibres more than enzymatic treatment at low consistency (1%).[56] The pore volumes were further increased by utilizing enzymes in high consistency treatment. This suggests that the mechanical action damages and breaks the tight structure of the cell wall and allows the enzymes to penetrate the cell wall structure and thereby have better access to the fibre components.[56]

Rahikainen et al. studied the effect of the cellulase structure at high consistency treatments.[56] The smallest enzyme was found to be also the most efficient and increase the pore volume best of the tested cellulases which support the assumption of enzyme penetration to the cell wall. With the smallest enzyme, the high consistency treatment was noticed to enhance the enzymatic action over six-fold when compared to the low consistency treatment. The most effective changes on fibre porosity and morphology have been shown to occur at consistencies between 15–25%.[66]

3 Determination of cellulose molar mass

3.1 Introduction

Polymeric materials like cellulose usually consist of molecular chains with varying lengths and molar masses. The chains form a distribution that contains different amounts of chains with certain lengths. Therefore the molar mass of polymeric material cannot be expressed as one absolute value but the molar mass distribution is illustrated by calculating different average values. The commonly calculated averages are defined as:

Number average molar mass

$$M_n = \frac{\sum n_i M_i}{\sum n_i} \quad (1)$$

Weight-average molar mass

$$M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \quad (2)$$

z-average molar mass

$$M_z = \frac{\sum n_i M_i^3}{\sum n_i M_i^2} \quad (3)$$

where n_i is the number of chains that have a molar mass of M_i. The polydispersity index (PD) describes the broadness of the molar mass distribution and is defined as

$$PD = \frac{M_w}{M_n} \quad (4)$$

PD increases with broadening molar mass distribution. A monodisperse sample of polymer chains with the same lengths has PD=1.

Since cellulose is widely used in many applications in pulp and paper, textile, food, and pharmaceutical industries, the information of molar mass and its distribution are needed in fundamental studies and optimisation of cellulose processing techniques. Commonly used methods to study cellulose molar mass are viscometry, light scattering, and size exclusion chromatography (SEC) of which SEC is the only one capable to determine the molar mass distribution.

3.1.1 Polymer dissolution

Viscometry, light scattering and size exclusion chromatography all require a fully dissolved sample for successful determination of the molar mass. Dissolution of a polymer depends on the Gibbs free energy of mixing $\Delta \mathrm{G}$ which is defined as

$$\Delta G = \Delta \mathrm{H} - \mathrm{T}\Delta \mathrm{S} \quad (5)$$

where $\Delta \mathrm{H}$ is the change in enthalpy, T is temperature and $\Delta \mathrm{S}$ the change in entropy. The dissolution process is spontaneous and occurs if the change in the Gibbs free energy of mixing has a negative value.[68] Entropy change upon polymer dissolution is always positive which is due to the increase in the number of possible conformations the polymer can adopt.[69,70] Still, the entropy gain in polymer dissolution is small because the monomers are linked to each other reducing the number of possible

states of the system.[69,70] When the molar mass of a polymer increases, it is more difficult to dissolve because the number of possible states decreases and the significance of the entropy term decreases.

Therefore, dissolution of a polymer strongly depends on the enthalpy of mixing. Secondary forces like van der Waals, electrostatic and hydrophobic interactions and hydrogen bonds occurring between the molecules in the system determine the sign and magnitude of the enthalpy term.[68] If solvent-solvent interactions and polymer-polymer interactions dominate, the solvent is poor and not capable to fully dissolve the polymer. In a good solvent, interactions between the polymer and solvent dominate dissolving the polymer and giving it swelled conformation. The better the solvent is the bigger is the hydrodynamic volume of a polymer. In a theta solvent, the polymer coil has ideal conformation and is in the edge of solubility as the interactions between the polymer segments are equal to the forces between the polymer and the solvent.[71] In general, the structural and chemical similarity between polymer and solvent favours the solubility.

3.1.2 Viscometry

Fast and simple viscosity measurements are widely utilized in the pulping industry to give an estimation of the decrease in cellulose molar mass resulting from the pulping or bleaching process.[72] The method is based on the determination of relative viscosity which is a ratio between viscosity of the sample solution and viscosity of the pure solvent. Relative viscosity is used to calculate intrinsic viscosity which is then utilized to determine the viscosity average molar mass. The relation between intrinsic viscosity of a dilute polymer solution and viscosity average molar mass is defined with Mark-Houwink-Sakurada equation as

$$[\eta] = KM_v^a \qquad (6)$$

where $[\eta]$ is the intrinsic viscosity of a dilute polymer solution, M_v is the viscosity average molar mass and K and a are empirical constants that are unique for a given combination of polymer, solvent, and temperature.[73,74] The value a describes the polymer conformation in the solution and is 0 for a sphere, 0.5 for ideal conformation, and 1.8 for a rigid rod.[74] Viscosity of cellulosic samples is often determined in copper ethylenediamine hydroxide even if the solvent is known to degrade oxidized cellulose and diminish the results.[72,75]

3.1.3 Light scattering

Static light scattering provides weight average molar mass based on the relationship between the intensity of light scattered by a dilute polymer solution and the molar mass of scattering polymers.[76] The basic equation of light scattering from a dilute solution with large molecules is

$$\frac{K^* c}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2 c \tag{7}$$

where K^* is

$$K^* = \frac{4\pi^2 (\frac{dn}{dc})^2 n_0^2}{N_A \lambda_0^4} \tag{8}$$

and c is the polymer concentration, $R(\theta)$ is the intensity of light scattered by a sample (Rayleigh ratio) in an angle θ, $P(\theta)$ is the ratio of the Rayleigh ratios at angle θ and at zero angle, A_2 is the second virial coefficient, $\frac{dn}{dc}$ is the specific refractive index increment of the polymer in a solution, n_0 is the refractive index of the solvent, N_A is the Avogadro's constant and λ_0 is the wavelength of the laser.[77] The refractive index increment corresponds to the change in the refractive index of the solution as a function of the polymer concentration. The second virial coefficient describes interactions between the polymer and the solvent and it has a value above zero when the polymer molecules prefer interactions with solvent molecules and a value under zero when interactions between the polymer molecules are energetically favoured.

Static light scattering is commonly performed by measuring the scattered light in many angels and sample concentrations which allows the creation of a Zimm plot which provides the M_w, A_2 and radius of gyration that describes the size of a molecule in solution.[78] Alternatively, when combined with a concentration detector, light scattering may be utilized as an on-line detector in size exclusion chromatography to determine the molar mass distribution which is utilized to calculate the molar mass averages.[76]

3.2 Size exclusion chromatography

Size-exclusion chromatography (SEC) is a type of liquid chromatography that separates molecules based on their hydrodynamic volume in solution. SEC is widely used to determine polymer molar mass distribution which is in turn used to calculate PDI and molar masses like M_n and M_w. The separation of samples of different hydrodynamic volumes is based on a column packed with porous polymer beads that retain the sample molecules according to their size.[79] Ideally, the separation is purely based on hydrodynamic volume and all enthalpic interactions between the sample and the surface of the

column should be minimised.[79] The first version of SEC was invented in 1955 by Lathe and Ruthven as they separated substances according to molar mass by using a starch gel containing column.[80] In 1959, the technique was further improved by Porath and Flodin who used synthetic dextran gels to pack the separation column.[81]

In size exclusion chromatography, the sample molecules travel through the column with constant eluent flow. The purpose of the pores in the column is to retain the samples and slow down their elution speed. Molecules with larger hydrodynamic volume elute faster since they are too large to enter most of the pores and therefore have a shorter route to travel. The chemically similar molecules with smaller molar mass have smaller hydrodynamic volume in the same solvent. The smaller molecules have a higher amount of possible pores to enter which increases their time in the column and they elute later. The size of the column pores determines the molar mass area it separates.[82]

After the column, the separated molecules are detected as the detectors monitor changes in the eluting solution. Common detecting techniques are refractive index (RI), ultraviolet (UV), infrared (IR) light scattering, and viscometry. Signals of RI and UV detectors give sample concentration versus the elution time which means that they are proportional to the sample concentration in the solution. RI detector is universal but less sensitive and its signal is related to the specific refractive index increment (dn/dc). UV detector is highly sensitive but works only with compounds with UV-absorbing functional groups. Light scattering detection is proportional to sample molar mass and concentration and viscometer is proportional to the product of molar mass and concentration to the power of the Mark-Houwink exponent. Peak area given by a detector is directly proportional to the injected amount of a sample and should be the same with the same detector, sample, and sample amount. Nevertheless, the response of a detector varies between different compounds and detector types based on the properties of the compound and the feature that the detector measures.[79]

Most of the detecting methods used with SEC require the creation of a calibration curve which is used to relate the elution time to the molar mass of the eluting samples in the used solvent and column. These relative methods include IR, viscometric, and UV detectors. The calibration curve for the column is created with a series of narrow polymer standards with known molar masses. Calibration is based on an assumption of the chemical similarity of the sample and the standard. Chemically similar polymers with the same molecular weights have the same hydrodynamic volume in the same solvent and therefore the same retention time.[83]

Column calibration by creating a calibration curve with known standards is not needed when using a light scattering detector such as MALS. However, the light scattering detector itself has to be calibrated so it cannot be referred as a completely absolute method. Calibration constant for the detector is

determined because the characterisation of the needed experimental constants, such as the power of the laser and the distance between the sample and the detector, is impossible with the required accuracy. Additionally, the detector requires precise dn/dc value and accurate knowledge of the sample concentration or a calibration constant of the RI detector for its determination. The signal of light scattering detector is related to the product of $(dn/dc)^2$ and molar mass.[79]

3.2.1 SEC calibration

A commonly used calibration standard with cellulosic samples is a series of narrow pullulan standards.[84–86] Pullulan has a similar linear structure as cellulose forming of D-glucopyranose units but its repeating units are linked to each other differently. Most of the repeating units in pullulan chain are linked by α(1→4) glycosidic bonds between the carbons 1 and 4 as in cellulose (β(1→4)) but every third bond is α(1→6) glycosidic bond allowing more rotational freedom for the chain.[87] Therefore cellulose has more rigid backbone than pullulan which leads to a higher hydrodynamic volume than pullulan with the same molar mass. Because of this, cellulose and pullulan have different Mark-Houwink constants and cellulose molar mass is overestimated with pullulan calibration.[88] Using conventional pullulan calibration, M_w is measured to be overestimated with a relative error of 55–90% when compared to the values obtained with multiangle laser light scattering (MALS).[89]

As a nearly absolute detector, MALS works without using an external calibration curve and therefore gives more reliable results if accurate concentration and dn/dc are known and therefore should be used whenever possible.[79] However, the error deriving from the use of pullulan standards can be decreased computationally if only an RI detector is available. Berggren et al. developed two methods to enhance results based on the pullulan calibration.[89] The first technique is based on a correlation between the molar mass determined using pullulan standards and MALS. This method only improves the molar mass values but doesn't correct the molar mass distribution. The second technique utilizes calculated cellulose-equivalent molar masses of pullulan standards. In addition to the molar masses, the second method improves also the molar mass distribution.

3.2.2 Dissolving cellulosic samples

Cellulosic fibres are difficult to dissolve and SEC analysis demands a completely dissolved sample. Accessibility of cellulose varies between its sources leading to differences in their solubility and receptivity for chemical modification. In general, cellulose samples have low accessibility mainly originating from the wide hydrogen bond network and the rigid fibrous structure of the fibres.[21] The

high abundance of intra- and intermolecular hydrogen bonds forms highly ordered crystal structures which are usually thought to be less accessible than the amorphous areas. The role of crystal structures in dissolution was long unclear.[32,90] Several contrary results showed either that the degree of crystallinity affects the sample solubility or that it doesn't have any impact.[91–94] Nevertheless, Ghasemi et al. proved in their phenomenological modelling that the degree of crystallinity affects dissolution but the advantage of reducing the degree of crystallinity can be predicted based many case-by-case conditions like the initial crystallinity and the quality of the solvent.[90] Similarly as for all polymers, high molar mass of cellulose hinders its dissolution.[93] Additionally, cellulosic samples often contain hemicelluloses and lignin which contribute to dissolution.

There are only a limited amount of SEC compatible solvents capable to dissolve cellulose. Aqueous metallo-complexes, like copper ethylenediamine and cadmium tris(ethylenediamine), are conventional cellulose solvents used in other analyses but unfortunately, they degrade oxidized cellulose due to their alkaline nature.[75] Despite that, copper ethylenediamine is still used to dissolve cellulosic samples in viscosity measurements as a part of standard methods in the industry.[95,96] Additionally, detection utilizing light scattering is not possible because of the intense colour of these metallo-complexes. A widely used solvent for cellulose processing is N-methylmorpholine-N-oxide (NMMO). It dissolves cellulose only at high temperatures and can chemically modify the fibres and is, therefore, less used in analytics.[97,98] Ionic liquids are great cellulose solvents and their potential as SEC eluents has been studied during the past decade.[99,100] However, elevated column pressures deriving from their high viscosity and relatively expensive cost have prevented their wide use as SEC solvents.[101] Additionally, aqueous NaOH solutions combined with additives such as zinc oxide or urea have been studied to dissolve cellulose for different applications.[102] After all, aqueous NaOH/urea solution is shown to degrade cellulose.[103]

After derivatisation many cellulose samples can be soluble in some conventional organic solvents. This is usually done by derivatising cellulose to cellulose carbamate for example with phenyl isocyanate and then utilizing commonly used tetrahydrofuran as a solvent and eluent.[101,104] However, low molar mass cellulose might be lost during the derivatisation process and samples containing large quantities of lignin are not soluble.[101]

Nowadays, lithium chloride/*N,N*-dimethylacetamide (LiCl/DMAc) is considered as the most suitable cellulose solvent for SEC.[105] It dissolves most of the cellulosic samples after activation procedures but some samples like softwood kraft pulp require additional derivatisation to improve the dissolution.[106] The solubility of cellulose in LiCl/DMAc was first noticed in 1979 by McCormick and Lichatowich and a few years later by Turbak et al. with minor or no degradation.[107,108] Later studies supported this

presumption of a rather stable system but a minor decrease in viscosity was noticed after 30 days of dissolution.[84,109] The colourless of LiCl/DMAc enables its use with MALS detection. Downsides of the solvent system are laborious sample activation procedure before the dissolution, poor solubility of softwood kraft pulps without derivatisation, and the use of LiCl. The high salt concentration increases the solvent viscosity which leads to higher applied pressure in the chromatographic system and complicates operating SEC.[106] Also, chloride ions have a corroding effect towards the SEC instrument with a presence of a small amount of water limiting the choice of the column material.[101]

A similar but less used solvent system for SEC is lithium chloride/1,3-dimethyl-2-imidatzolidinone (LiCl/DMI).[110] LiCl/DMI is less toxic than LiCl/DMAc and it dissolves many cellulosic samples like softwood kraft pulp without derivatisation, unlike LiCl/DMAc.[101,111] However, dissolving in LiCl/DMI requires many activation procedures as well and is not simpler to use than LiCl/DMAc. Besides, the viscosity of LiCl/DMI is higher than that of LiCl/DMAc which is a disadvantage in SEC.[112]

Dissolution of cellulose I (natural or pulped cellulose) and II (regenerated or alkali-treated) allomorphs differ from each other.[105] Focus of this literature part is within methods of dissolving cellulose I because of its relevance to the experimental section. In addition to the crystal structure, also the source of cellulose affects its dissolving properties because of differences in fibre morphology, chemical composition and their distribution in the fibres, and molar masses of components. Also, pulping modifies the properties of the samples by lowering DP of cellulose and removing most of the lignin and part of hemicellulose which contribute to the solubility of sample. For example, sulphite pulps are so strongly processed and have high content of low DP cellulose that they are soluble in LiCl/DMAc regardless of being from soft- or hardwood source.[85]

The difference in solubility of softwood and hardwood kraft pulps originates from the different hemicellulose composition and content which affects cellulose accessibility.[113] Hardwood hemicellulose is mostly composed of xylan and it has many acetylated groups while softwood hemicellulose has a higher portion of galactoglucomannan.[29] Galactoglucomannan has been shown to associate with cellulose and form a gel in LiCl/DMAc which hinders the complete dissolution of cellulose.[114,115] Removing of hemicelluloses is difficult since at the end of kraft cooking some of the dissolved and deacetylated hemicelluloses re-precipitate on the top of the cellulose fibres. Also, lignin is mostly removed in kraft pulping but the residues hinder the dissolution and even fully bleached pulps are not fully soluble.[114] Sjöholm et al. noticed different elution profiles of softwood and hardwood samples supporting the assumption of softwood hemicellulose binding to cellulose with hydrogen bonds.[116] Nevertheless, the elution profiles of softwood and hardwood kraft pulps look similar after

derivatisation and better dissolution of the softwood sample.[106] The association between cellulose and galactoglucomannan was observed to increase with decreasing amount of galactose.[116]

3.2.3 Dissolution mechanism of cellulose in LiCl/DMAc

Inter- and intramolecular hydrogen bonds of cellulose determine together with van der Waals interactions how cellulose swells and dissolves.[117,118] These changes have been studied utilizing model compounds with isotopic labeling in liquid- and solid-state nuclear magnetic resonance (NMR) spectroscopy that allows detecting breakage of hydrogen bonds in cellulose and formation of new bonds between the swelling agent or solvent.[117,119,120] These studies employ simplified model compounds and focus on hydrogen bonds and interactions between the solvent and solute without considering the impact of cellulose morphology or the presence of hemicellulose or lignin. Therefore, they are not strictly comparable to the dissolution of many actual samples like pulps.

The dissolution mechanism of cellulose in LiCl/DMAc has been studied since 1985 when the first study was published by McCormick et al.[109] Over time, the proposed mechanism of the dissolution has been updated a few times.[109,120–122] All these studies agree that intermolecular hydrogen bonds of cellulose are broken when the hydroxyl protons form hydrogen bonds with the Cl-anions. Breaking intermolecular hydrogen bonds and creating new ones with Cl-anions allows the chains to disperse in the solvent system. The role of Li-cation in the dissolution mechanism varies between the proposed mechanisms. The latest study is by Zhang et al. who studied cellulose dissolution in LiCl/DMAc utilizing cellobiose as a model compound.[120] They suggested that Li-cations are solvated by free DMAc molecules once the LiCl ion pairs have split in the formation of the hydrogen bonds between cellulose and Cl-anions. According to their study, cellulose forms hydrogen bonds with the Cl-anions rather than with DMAc molecules in the presence of excess LiCl. Proposed dissolution mechanism of cellulose in LiCl/DMAc by Zhang et al. is illustrated in Figure 7.

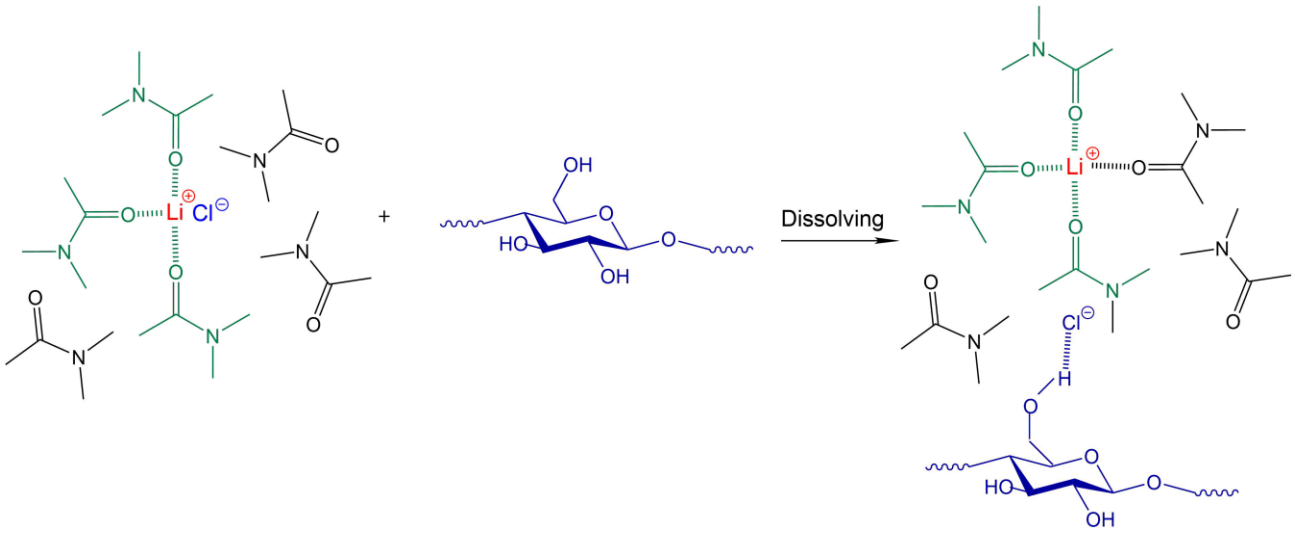


Figure 7. Dissolution mechanism of cellulose in LiCl/DMAc proposed by Zhang et al.[120]

3.2.4 Methods to improve the dissolution of cellulosic samples in LiCl/DMAc

Cellulose dissolution in LiCl/DMAc is enhanced and accelerated if the sample is activated before dissolution. The activation process modifies the supramolecular structure and changes the conformation of cellulose chains which makes cellulose hydroxyl groups more accessible for the solvent molecules.[123] There is a wide group of mechanical, chemical, and biological means to activate cellulose but unfortunately, most of these are cellulose degrading or not efficient. A common and not degrading activation method used prior to dissolution to DMAc is a solvent exchange process.

The solvent exchange process increases cellulose molecular mobility and removes dissolution hindering water.[123,124] The process is usually started by swelling the sample in a suitable solvent like water. Then, the sample is commonly transferred to another solvent like methanol or acetone before swelling it in DMAC which is followed by finally dissolving the sample to LiCl/DMAc.[125–127] The exchange is often performed by filtration or centrifugation and the same solvent is often changed to fresh one couple of times before changing the type of the swelling agent. Sample swelling in intermediate solvents is slow and normally the whole solvent exchange process lasts a couple of days. The downside of the solvent-exchange process is unavoidable weight losses.[112] This makes accurate controlling and determining cellulose concentration impossible which would contribute accurate MALS detection.

An additional way to improve pulp dissolution is by partial derivatisation of cellulose and hemicellulose hydroxyl groups. Derivatisation decreases the amount of possible inter- and intramolecular hydrogen bonds and allows easier dissolution.[105] A common derivative, cellulose carbamate can be produced by derivatising cellulose either with ethyl or phenyl isocyanate directly in LiCl/DMAc (Figure 8). With ethyl

isocyanate, the degree of substitution is nearly two which leads the molar mass to increase with a factor of 1.87 at maximum.[105] Despite the increase in molar mass, ethyl isocynate derivatisation does not cause a significant change in the sample hydrodynamic volume. There is no change or only a minor change in the molar mass distributions of ethyl isocyanate derivatised hardwood sample or pullulan standard.[105,128] This is probably due to the similar size of the ethyl carbamate substitutes and the solvent molecules.[128]

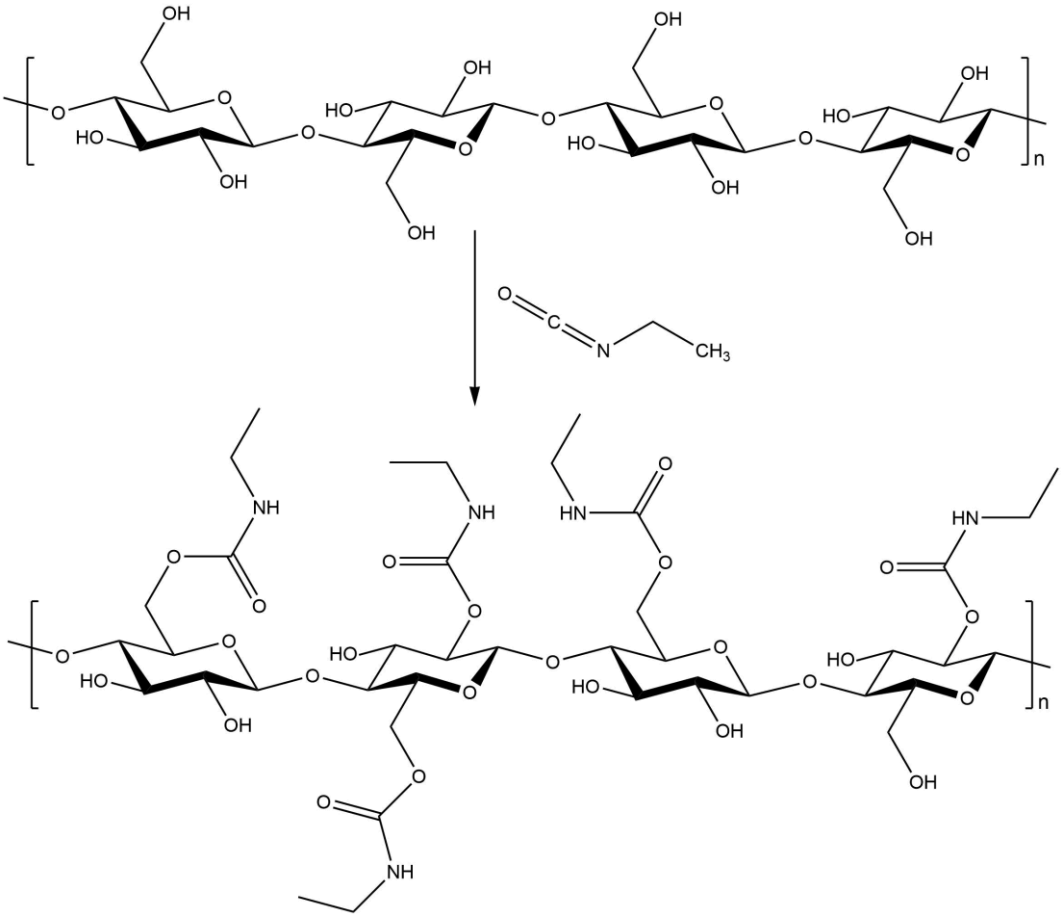


Figure 8. Cellulose derivatisation with ethyl isocyanate.

Derivatisation of softwood kraft pulp also changes the shape of molar mass distribution given by size exclusion chromatography resulting from better dissolution of the sample.[128] Usually, SEC chromatograms of pulp samples show a bimodal distribution where the larger high molar mass peak corresponds mainly to the distribution cellulose and the lower molar mass peak represents the fraction of lignin and hemicellulose.[116] Nevertheless, cellulose and hemicellulose are not well separated in underivatised softwood kraft pulp samples yielding a wide unimodal chromatogram. This is because of softwood galactoglucomannan which is shown to elute over the entire molar mass range[116] and hinder cellulose dissolution in general.[114] However, by derivatising softwood kraft sample, the bimodal

chromatogram and more correct estimation of the molar mass distribution can be achieved as the derivatisation disturbs the association of cellulose and hemicellulose and allows them to elute separately.[128] In addition, the ratio between high and low molar mass distributions of derivatised softwood samples correspond close to the actual chemical composition of the pulp.[128]

Heating of cellulose is often used as an activation method during swelling or dissolving in DMAc or LiCl/DMAc but it may lead to cellulose degradation. Degradation is notable especially above 85 °C as N,N-dimethylketene semiaminal, and N,N-dimethylketeniminium ions become present in LiCl/DMAc.[129] N,N-dimethylketeniminium ions are highly reactive and able to cleave glycosidic bonds. Also, microwave-assisted heating and irradiation are unfavourable ways of actvating cellulose due to sample degradation.[130–132] Additionally, some chemical activations like alkaline treatment or chemical reduction with alkaline reagents can cause degradation to oxidized samples.[36,133]

Examples of other pulp activation methods include milling, steam explosion, ultrasonic treatment, and freeze-drying. Milling, steam explosion, and ultrasonic treatment can degrade cellulose and their excessive use should be avoided[134–139] whereas freeze drying of cellulose swollen in LiCl/DMAc through the solvent exchange process can be performed without degradation.[140] However, it has not been found to be necessary for the dissolution but it allows preparing accurate concentrations when the dry already activated sample is later dissolved in LiCl/DMAc.[140,141]

EXPERIMENTAL

4 Introduction

Enzymatic modification of paper grade softwood kraft pulp was studied at high and low pulp consistencies. The target was to decrease the degree of polymerization (DP) of cellulose and lower the quantity of hemicellulose. The purpose was to examine enzymatic pulp treatment as a way to modify paper grade kraft pulp to be a suitable raw material for the future textile industry where ionic liquids could be exploited in cellulose dissolution and creation of wood-based regenerated fibres.

Humicola insolens Cel45A (*Hi* Cel45A) endoglucanase was produced as a prospective tool for the pulp modification to produce adjusted raw material for the textile application. The optimal pH and temperature conditions of the enzyme were determined in high consistency pulp treatments. Unfortunately, the endoglucanase preparation was found to be unstable and the actual pulp modifications were performed with commercial endoglucanase.

The pulp modifications were performed utilizing cellulose degrading commercial endoglucanase together with hemicellulose degrading mannanase and xylanase. Different combinations of these enzymes were studied both at high (20%) and low (3%) pulp consistency because recently several studies suggest that enzymatic pulp modification enhances at high pulp consistency but systematic studies on the topic are lacking.[56,64–66]

After the treatments, the modified pulps were analysed. The amount of solubilised sugars from the treatments were determined using enzymatic hydrolysis together with dinitrosalicylic acid reagent and the composition of the solubilised sugars was analysed utilizing mild acid hydrolysis before high performance liquid chromatography. The sample pulps were studied with optical microscopy and their intrinsic viscosities were measured. Changes in molar mass distributions of each sample were analysed using size exclusion chromatography (SEC). Additionally, challenges with cellulosic and especially softwood kraft pulp samples related to sample dissolution and SEC are discussed.

5 Materials

Bleached softwood kraft pulp from Metsä Fibre was received from a Finnish wood mill as dry sheets. For enzymatic pulp treatments, 4 different enzyme preparations were used, two endoglucanase, one mannanase, and one xylanase rich preparations. The enzymes were dosed based on protein concentration as mg protein per gram of dry pulp. *Humicola insolens* Cel45A endoglucanase was produced at VTT in *Tricoderma reesei* synthetic expression strain. Endoglucanase activity towards

carboxymethyl cellulose and protein concentration based on the Lowry method were determined as a part of the work.[142,143]

In addition to the VTT produced endoglucanase, commercial endoglucanase rich Fibercare R preparation from Novozymes (Bagsværd, Denmark) was used. Properties of the preparation had been determined earlier.[66] Endoglucanase activity of the enzyme preparation was determined to be 2760 nkat/ml assayed at pH 5 using hydroxyethylcellulose (HEC) as a substrate. Negligible xylanase activity of 4 nkat/ml was assayed from the preparation at pH 5. Protein concentration of the commercial product was determined after protein precipitation and re-dissolution using a commercial kit (DC protein assay, Bio-Rad Laboratories) which is based on the Lowry method.[143] Based on the enzyme qualities and behaviour, the endoglucanase resembles endoglucanases from glycoside hydrolase family 45.

Pure hemicellulases used in the work were readily available at VTT and their protein concentrations were known from previous works.[144,145] Mannanase and xylanase were both enzymes of *Trichoderma reesei* fungus. *Trichoderma reesei* Man5A is part of GH5 family and it initiates the hydrolysis of (1→4)-β-D-mannosidic linkages internally in mannans and heteromannans. Man5A has a pH-optimum at 3.5–4 and has a residual activity of 72% at pH 6. It is thermally stable for 24 h at 30 °C. After incubation at 80 °C for half an hour, 80–85% of the activity is still left.[144] *Trichoderma reesei* Xyn11A is part of glycoside hydrolase 11 (GH11) family and it initiates the hydrolysis of (1→4)-β-D-xylosidic linkages internally in xylan. Xyn11A has a pH-optimum at 5–5.5 and it retains its activity at room temperature for 24h between pH 3–8. It is thermally stable for 24 h at 45 °C.[145]

6 Cold-disintegration of the pulps and quantification of the pulp composition and the dry matter content

Pulp sheets were shredded and soaked in reverse osmosis water (2 kg/20 l) for 2 hours followed by transfer to a pilot scale mixer where total volume was adjusted to 50 l. During disintegration, the metal pegs of the device were programmed to rotate 300 rpm for 15 min. Excess water was removed on a screen followed by pulp homogenisation.

Chemical composition of the pulp was determined with high performance liquid chromatography after a two-step sulphuric acid hydrolysis following the protocol published by NREL.[146] Dry matter content was analysed gravimetrically heating three replicate samples in a 105 °C oven.

7 Characterization of the produced endoglucanase

7.1 Protein quantification

Humicola insolens Cel45A endoglucanase was produced using an engineered *Trichoderma reesei* strain (M3022). The production was done in a 20L fermentor on a culture media containing glucose and yeast extract. The fermentation broth was down-stream processed and concentrated using ultrafiltration prior to further analysis and use.

The total enzyme concentration of *Hi* Cel45A in the growth solution was determined based on the method created by Lowry et al.[143] The quantification was done using a commercial Bio-Rad DC Protein Assay kit which is a modified version of the original protein assay. The determination is based on a two-step reaction that leads to detectable colour development in the sample solution.

Before the analysis, the protein sample was precipitated to remove possible residues from the growth solution. First, 50 µl of sample solution was diluted with 350 µl of 50 mM sodium phosphate buffer (pH 6). 250 µl of this dilution and 1000 µl of cold acetone (-20 °C) were added to an Eppendorf, vortexed and stored in a freezer (-20 °C) for overnight. The next day, the precipitated sample was centrifuged with 14 000 x g for 10 minutes. The supernatant was removed and the rest of the acetone was let to evaporate in a fume hood for 30 minutes. The remaining protein was dissolved into 250 µl of aqueous solution that contained 2 wt% of Na_2CO_3 and 0.4 wt% of NaOH for every 1000 ml of deionized water. The same aqueous salt solution was used to dilute the sample for the actual Lowry protein assay.

The calibration curve was created using aqueous Bovine serum albumin (Sigma-Aldrich A-8022) standard solution. The assay was started by adding 100 µl of sample and standard dilutions into separate test tubes followed by the addition of 500 µl of alkaline copper tartrate solution and vortexed. The cupric ions formed chelates with peptide bonds of the protein and were reduced to cuprous ions. Then, 4 ml of dilute Folin reagent was added and vortexed which led to a reduction of phosphomolybdotungstate to molybdenum blue by the cuprous ions. A blank sample for the spectrometer was prepared in the same manner but replacing the protein solution with 100 µl of deionised water. The samples were incubated at room temperature for 15 minutes to allow reduction of the Folin reagent that generates the detectable colour. Intensity of the colour depends on the number of cuprous ions and therefore the concentration of the protein.[147] The absorbances were read at 750 nm using Hitachi U-2000 Spectrometer.

7.2 Gel electrophoresis

Purity of the produced enzyme *Hi* Cel45A was studied with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which separates proteins based on their mass. The method is developed by Ulrich K. Laemmli.[148] It utilizes sodium dodecyl sulphate (SDS) which unfolds the tertiary and secondary structures of proteins. Additionally, SDS binds to the surface of proteins covering the intrinsic protein charges and simultaneously giving a new negative charge to the protein due to the sulphate group in SDS. This gives a similar mass-to-charge ratio to all proteins allowing separation almost purely by mass.

The sample solutions were prepared by adding three volume units of protein solution to one volume unit of SDS-MIX solution. The aqueous SDS-MIX contained SDS, glycerol, β-mercaptoethanol, and Bromphenol blue and tris(hydroxymethyl)aminomethane and HCl for adjusting pH. The samples were boiled for 5 minutes and sampled into polyacrylamide based Criterion TGX Stain-Free Precast Gels (Bio-Rad). As a running buffer was used a 1:10 dilution of a ready buffer concentrate (Bio-Rad) containing tris(hydroxymethyl)aminomethane, glycine, and SDS. The gel was run in Criterion Cell (Bio-Rad) with a constant voltage of 250 V powered by PowerPac Basic (Bio-Rad). The gel was imaged with UV-light in Gel Doc EZ Imager (Bio-Rad) using Image Lab v.6.0.1 -software.

7.3 Enzyme activity determination with carboxymethyl cellulose substrate

Activity of *Hi* Cel45A endoglucanase was determined utilizing a carboxymethyl cellulose (CMC) substrate with a similar principle as the IUPAC standard assay procedure to measure endoglucanase activity.[142] The enzyme activity was measured based on the amount of liberated free reducing chain ends at a certain time. The CMC substrate solution was prepared by dissolving 1 % of carboxymethyl cellulose sodium salt (low viscosity CMC sodium salt, DS 0.65–0.9, Sigma-Aldrich) into 0.05 M sodium citrate buffer. The same buffer was also used in a dilution of the protein sample and standard. The calibration curve was prepared using a glucose standard of 0.01 M. Enzyme blank samples and a zero sample for the spectrometer were prepared.

The analysis was started by adding 450 µl of substrate solution to empty test tubes collected for the samples, standards, enzyme blanks, and zero samples. The tubes were placed in a water bath of 50 °C. When the CMC solutions had reached 50 °C, 50 µl of each enzyme dilution was added to their tubes, vortexed and directly put back to the water bath. Simultaneously, all the other test tubes stayed in the water bath containing only the substrate solution.

After ten minutes of incubating with the sample solutions, 750 µl of dinitrosalicylic acid (DNS) reagent was added to each tube followed by immediate vortexing and lifting the tube from the water bath. The aqueous DNS contained dinitrosalicylic acid (1%), NaOH (1.6%), and potassium sodium tartrate (30%). DNS worked as a stopping reagent for the reaction and it reacted with the reducing sugars that were produced by the enzyme. A light-absorbing 3-amino-5-nitrosalicylic acid was formed in this reaction.

The standards, enzyme blanks, and a zero sample were prepared similarly by adding 50 µl of each solution into test tubes but only after adding the DNS reagent. The enzyme blanks samples were prepared to study the original sugar amount in the original sample enzyme solution. The same enzyme dilutions that were used to prepare the proper samples were used to create these enzyme blanks. The zero sample for the spectrometer was prepared using the buffer.

All the test tubes were placed to boiling water for five minutes and after cooled down. The absorbance of each sample was read at 540 nm using a Hitachi U-2000 Spectrometer.

8 Enzymatic pulp treatments

Enzymatic pulp treatments were performed at high and low consistency of 20 and 3% of pulp dry weight respectively. The high consistency treatments were done utilizing a farinograph mixer with two z-shaped mixing blades and the low consistency treatments were done in a beaker with a flat and wide blade mixer.

8.1 High consistency treatments

The high consistency treatments were performed using a farinograph mixer (Brabender, Germany) that had a thermostatic water chamber for temperature adjusting. The mixer had a two-part mixing container with one mixing blade in each to rotate pulp between the two parts (Figure 9). The treatments were done either with 50 g of dry pulp and 20% consistency or 60 g of pulp and 24% consistency and they were all buffered to the wanted pH using a buffer. The buffer, enzyme solutions, and deionised water were mixed in a spray bottle and sprayed evenly on the cold disintegrated pulp prior transferring the pulp to the preheated farinograph. Mixing was started with a speed of 25–30 rpm.

After two minutes of mixing, the pH of the mixture was measured and mixing was continued. After two hours of mixing, the pH of the mass was measured and the pulp was collected. The enzyme activity

was terminated by adding boiling water to decrease the pulp concentration to 10%. To ensure the termination, the pulp was incubated at boiling water for 20 minutes. After boiling, the sample was vacuum filtrated with 60 µm filtration cloth. A filtrate sample was collected to determine the dissolved sugars and oligosaccharides (yield loss). The pulp sample was washed with cold deionised water and homogenized with a Kenwood mixer.

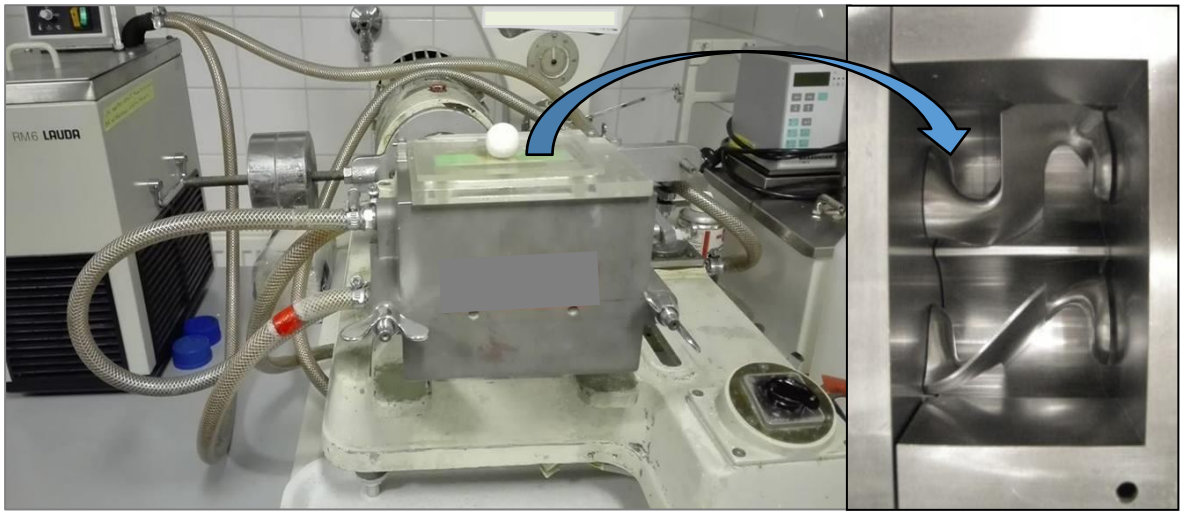


Figure 9. Farinograph used for mixing at high consistency.

8.2 Low consistency treatments

The low consistency (3%) treatments were performed with 20 g (dry weight) of cold disintegrated pulp. The treatments were buffered with 100 mM ionic strength to the wanted pH. The buffer solution was heated in a beaker to the reaction temperature before the pulp was suspended in it with a kitchen mixer. The beaker containing the pulp and the buffer was placed to a water bath of 50 °C and constant mixing of 25–30 rpm was started with a blade mixer. A diluted enzyme solution was evenly added to the mixture while the mixing was on. After two minutes of mixing, the pH was measured. Then the mixing was continued until two hours had passed since adding the enzyme dilution.

After two hours, the mixing was turned off and the pH of the mass was measured. The pulp was vacuum filtrated and a filtrate sample was collected to measure the number of dissolved sugars and oligosaccharides. The enzyme activity in the pulp was terminated by adding boiling water on the pulp until the concentration of the pulp was 10%. Then, the pulp and the filtrate sample were incubated at boiling water for 20 minutes to terminate the enzyme activity. After boiling, the pulp was vacuum

filtrated with 60 µm filtration cloth. The pulp was washed with cold deionized water and homogenized with a Kenwood mixer.

8.3 Temperature and pH optima of Hi Cel45A in high consistency pulp treatment

Temperature and pH optima of *Hi* Cel45A endoglucanase for pulp substrate were determined at high consistency. First, pH optimum was determined at 50 °C, and then temperature optimum was determined at the discovered pH optimum. All treatments had constant *Hi* Cel45A endoglucanase dose of 0.017 mg/g. The treatments were done using 60 g (dry weight) of cold disintegrated pulp and 24% pulp consistency.

The effect of the pH was studied by performing seven pulp treatments at different pH values by utilizing sodium acetate and sodium phosphate buffers between pH 5 and 8 (Table 1). Ionic strength deriving from the buffers were kept relatively low as 105 mM. High ionic strength can be harmful to enzyme activity by effecting enzyme adsorption or changing its conformation.[149,150] Additionally, the buffers were utilized in the limits of their buffering capacity. Therefore, most of the pH values measured from the pulp mixtures differ from the targeted pH values that the buffers originally had (Table 1). In general, the differences increase when the targeted pH value gets further of the buffer pKa value. However, small deviations from the targeted pH values did not prevent performing a series of treatments with varying pH conditions and the determination of the pH optimum. Therefore it was not necessary to utilize other buffers in the pH areas where the buffering capacity of sodium acetate and phosphate buffers were limited.

Table 1. Buffers, pH values of the buffers, and the measured pH values in the beginning and end of the high consistency treatments with *Hi* Cel45A.

Treatment no.	Buffer	Buffer pH	Measured pH in the beginning	Measured pH in the end
1	sodium acetate	5.0	4.9	5.1
2	sodium acetate	5.5	5.4	5.9
3	sodium phosphate	6.0	6.2	6.3
4	sodium phosphate	6.5	6.7	6.5
5	sodium phosphate	7.0	7.0	7.0
6	sodium phosphate	7.5	7.4	7.4
7	sodium phosphate	8.0	7.6	7.7

Temperature optimum of *Hi* Cel45A in pulp treatment was determined by performed a series of five treatments between temperatures 45 and 65 °C. All treatments were performed utilizing 105 mM sodium phosphate buffer at pH 6.5.

8.4 Combination of endoglucanase and hemicellulases in high and low consistency pulp treatments

Combinations of endoglucanase and hemicellulases in enzymatic treatment of softwood kraft pulp were studied at high (20%) and low (3%) consistencies. Temperature and pH used in the treatments were selected as a compromise based on the optima of the utilized enzymes. All the treatments were performed at 50 °C and buffered with 100 mM pH 6 phosphate buffer. Similar series of seven treatments were conducted both at high and low consistency. A constant dose of 0.02 mg of commercial endoglucanase rich preparation was used in all treatments. However, the dosage of mannanase (*Tr* Man5A) and xylanase (*Tr* Xyn11A) were varied for each treatment. The dosage of each enzyme in all seven different combinations is shown in Table 2.

Table 2. Enzyme doses of seven separate treatments which were all performed both at high (20%) and low (3%) consistency for comparison.

Treatment	Commercial endoglucanase (mg/g)	Mannanase (*Tr* Man5A) (mg/g)	Xylanase (*Tr* Xyn11A) (mg/g)
A	0.02	-	-
B	0.02	0.1	-
C	0.02	0.5	-
D	0.02	-	0.1
E	0.02	-	0.5
F	0.02	0.1	0.1
G	0.02	0.5	0.5

9 Analysis of the pulps

9.1 Viscometry

The intrinsic viscosity of the pulps was determined in copper ethylenediamine (CED) according to ISO 5351-1 using a PSL Rheotek instrument (Poulten, Selfe & Lee Ltd, UK).[96] The CED solvent was prepared by saturating 1.00 M CED with copper(II) hydroxide. Viscosities of the solvent and the sample solution are so different that a special calibration viscometer was used in the calibration. The flow times of

deionised water, glycerol solution (65 wt%), and 0.50 M CED solution, which was prepared by diluting the earlier prepared 1.00 M CED solution with deionised water, were measured using this calibration viscometer. Additionally, the flow time of glycerol solution was determined using the actual sample viscometer. All the calibration solutions were heated to 25 °C and their viscosities were determined in duplicates at a constant temperature of 25 °C.

Concentrations of the sample solutions were between 0.003–0.005 g/ml and they were selected based on preliminary measurement. Preparation of the sample solutions was started by adding the weighted sample and 25.0 ml of deionised water in a polyethylene bottle. After mixing, 25.0 ml of 1.00 M CED solution was added and the remaining air in the bottle was removed. The solution was mixed until all sample was dissolved and the bottle was placed in a water bath of 25 °C. Viscosities of the samples were determined in duplicates at a constant temperature of 25 °C. Standard deviation of the intrinsic viscosity replicates was within ±10 ml/g.

9.2 Size-exclusion chromatography

9.2.1 Dissolution of the samples

The samples were dissolved in 8% LiCl/DMAc by a method developed by Berthold et al. including solvent exchange and a direct ethyl isocyanate derivatisation.[128] The pulp samples were freeze-dried before dissolving. Three parallel solutions were prepared from each sample. Swelling of the samples was started by suspending them in deionised water. After soaking, the samples were centrifuged and the excess water was removed and replaced with fresh water. This was repeated twice before adding water once more and placing samples to a water bath of 60 °C for 3 hours. After the heating period, water in the samples was exchanged to methanol. The samples were placed into a fridge for overnight.

On the next day, methanol in the samples was exchanged to fresh methanol twice and placed back to the fridge for overnight. The next day, the methanol was removed and replaced with DMAc. After soaking, the samples were centrifuged and the solvent was removed. This was repeated twice before the samples were transferred to new vials with 8% LiCl/DMAc. The samples were set under nitrogen atmosphere and ethyl isocyanate was added for the derivatisation. The samples were left at ambient temperature with magnetic stirring for five days.

After derivatisation, the solutions were diluted with DMAc and the excess reagent was quenched with an addition of methanol. The samples were mixed with magnetic stirrers for overnight. On the next day, 1 ml of the sample solution was mixed with 3 ml of DMAc giving a final concentration of 0.8% LiCl. All the samples were filtered with a 0.45 µm PTFE filter before the analysis.

9.2.2 SEC-measurement

Size exclusion chromatography measurements were performed using a precolumn with two PL gel MiniMixed A columns at 80 °C. The eluent was 0.8% LiCl/DMAc with an elution speed of 0.36 ml/min. The elution was detected with two detectors, Waters 2414 Refractive index (RI) and Malvern 20 -angle MALS detector. In the case of RI detection, the molar mass distributions were calculated against 8 x pullulan (6 100–708 000 g/mol) standards and analysed with Waters Empower 3 software. In addition to the conventional pullulan calibration, the molar masses of pullulan standards were converted to correspond the molar masses of same size cellulose chains based on the method by Berggren et al.[89] The equation used was $MM_{cellulose} = q \times MM_{pullulan}^{p}$ were q was 12.19 and p was 0.78. The constants were found from the data in their report by a least-squares method. MALS detector was calibrated with pullulan P-50 standards of M_w = 48 800 kDa with a polydispersity of 1.07 and dn/dc value of 0.163 was applied. MALS results were calculated using OmniSEC (5.1) software. All the results are presented as plots of the differential mass fractions plotted against the logarithm of molar mass. The chromatograms were drawn based on calibration with cellulose-equivalent molar masses of pullulan.

9.3 Determination of dissolved sugars and oligosaccharides

The amount of cleaved oligo- and monosaccharides from the pulp was determined from the filtrate sample that was taken after processing and filtrating the pulp. The filtrate sample contained sugars, disaccharides, and other larger carbohydrates like oligosaccharides. Their combined weight is the same as yield loss of the process and it was determined by hydrolysing them to simple sugars and quantifying the amount of reducing sugars. This was done in two ways; by utilizing enzymatic hydrolysis together with dinitrosalicylic acid (DNS) assay of reducing sugars and by mild acid hydrolysis with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Quantifying reducing sugars with alkaline DNS-reagent that contains potassium was developed by Sumner and Noback.[151] The aqueous DNS contained dinitrosalicylic acid (1%), NaOH (1.6%), and potassium sodium tartrate (30%). To each sample dilution of 1 ml was added 1.5 ml of DNS-reagent and vortexed. The zero sample for the spectrometer was prepared with 1 ml of distilled water and 1.5 ml of DNS-reagent. Glucose standard was used similarly to prepare solutions for calibration. The solutions were boiled for 5 minutes leading to a reduction of DNS to 3-amino-5-nitrosalicylic acid. The amount of formed 3-amino-5-nitrosalicylic acid is proportional to the number of reducing groups in the filtrate and can be quantified spectrometrically. The absorbance was measured at 540 nm using Hitachi U-2000 Spectrometer. The 3-amino-5-nitrosalicylic acid concentration in each dilution was

calculated against the standard solutions. This result was used to estimate the number of needed enzymes for hydrolysis.

Before repeating the quantification of the reducing groups, the filtrate sample was hydrolysed with an enzyme mixture containing cellulolytic and hemicellulolytic enzymes.[97] The enzyme mixture consisted of concentrates of *T. reesei* and *A. foetidus* and glucuronidase with a total enzyme activity of 20 nkat/ml. The needed amount of the mixture was estimated based on the preliminary result of the reducing sugars. The undiluted filtrate sample (1.5 ml) was added to a test tube followed by the required amount of the enzyme mixture. Sodium acetate buffer (100 mM, pH 5) was added until the total volume was 2 ml. The enzyme blank sample was prepared in the same way but replacing the undiluted sample filtrate with distilled water. The tubes were vortexed and lift in a water bath of 45 °C for 24 h. The enzyme hydrolysis was terminated by boiling the sample for 3 minutes. After the hydrolysis, monomeric carbohydrates were determined again using the DNS-reagent as previously.

The sugar composition of the yield loss was determined with HPAEC analysis. Before the analysis, the oligosaccharides in the filtrate sample were hydrolysed with mild (4%) sulphuric acid for an hour at 120 °C in an autoclave. The monomeric sugars were determined utilizing Dionex ICS-5000 liquid chromatography with Dionex CarboPac PA-20 guard and analytical columns and ICS-5000 electrochemical detector. The gradient system used in the detection was based on previously published protocol by Tenkanen et al. with a modification on the B gradient by decreasing the NaOH concentration to 10 mM.[152] The monomeric sugars were calculated to correspond to polymeric weights. Xylose and arabinose were considered to originate from arabinoglucuronoxylan, mannose, galactose, and amount of glucose corresponding to 1/3 of total mannose were considered to originate from galactoglucomannan and the rest of the glucose was considered to originate from cellulose.

9.4 Light microscopy

Structure of the kraft fibres was studied before and after enzymatic modification with light microscopy. Before imaging, the fibres were stained with 1% (w/v) Congo red aqueous solution by mixing equal volumes of pulp and the stain solution. After half an hour, 100 µl of dyed pulp solution and 100 µl of water were added to a microscope glass. The fibres were imaged with Olympus BX61 microscope equipped with Olympus WH10X-H oculars and Olympus ColorView12 camera using Analysis Pro 3.1-software.

10 Results and discussion

10.1 Raw material characteristics

Relative carbohydrate and lignin composition of the softwood kraft pulp was determined. The major components of the pulp were cellulose (81.6%), xylan (8.7%), glucomannan (8.7%) and klason lignin (1.0%). The pulp had an intrinsic viscosity of 860 ml/g.

10.2 Analysis of the *Hi* Cel45A

Humicola insolens Cel45A endoglucanase was produced at VTT because of its likely suitability for enzymatic pulp treatments. The produced enzyme belongs to the glycoside hydrolase family 45, it has a carbohydrate-binding module from family 1 and it catalyses cellulose hydrolysis with an inverting mechanism.[153–155] The structure of its catalytic core is presented in Figure 10. Effect of cellulase family and structure in high consistency pulp modification was studied in a work by Rahikainen et al. in which endoglucanase from glycoside hydrolase family 45 was found to be efficient to decrease the DP of pulp cellulose and viscosity.[56] Besides, the endoglucanase from structural family 45 was the best of the three studied endoglucanases to increase fibre porosity. This was suggested to originate from the small size of the enzyme (37 kDa, from *Melanocarpus albomyces*) that allowed the enzyme to penetrate in the fibres.

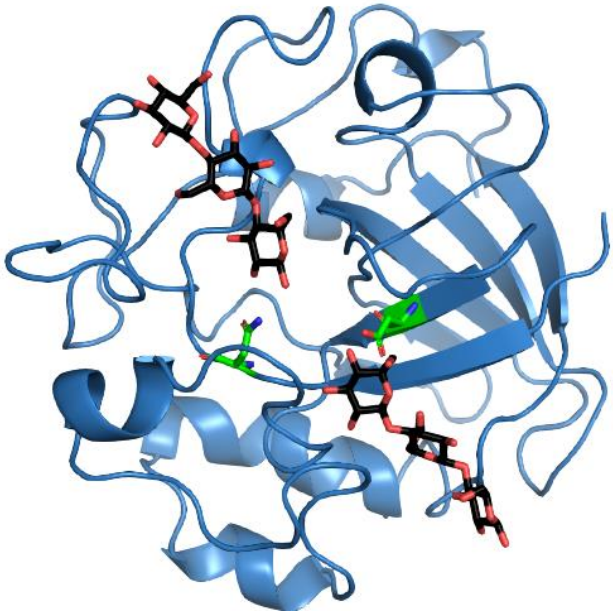


Figure 10. Structure of catalytic core of *Hi*Cel45A endoglucanase and a cellohexaose that has been hydrolysed to two cellotrioses. Active sites of the enzyme are coloured in green. Structure taken from RCSB Protein Data Bank based on study of Davies et al. and modified with PyMOL 2.3.3 -Molecular Graphics System(Schrödinger, LCC).[155,156]

Properties of the VTT produced *Hi* Cel45A endoglucanase were studied before the enzymatic pulp treatments. Based on the Lowry method, the protein concentration of the endoglucanase rich preparation was found to be 32.6 mg/ml. The activity of the *Hi* Cel45A against carboxymethyl cellulose was measured to be 19 500 nkat/ml. Low xylanase activity of 35 nkat/ml was assayed from the preparation at pH 5.

Molar mass and purity of the endoglucanase were measured using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Endoglucanase showed a wide band in the gel at 43 kDa similarly as in other studies of *Hi* Cel45A.[157,158] Additionally, the SDS-PAGE showed a wide faded band at 75 kDa and two faded narrow bands at 37 and 25 kDa (Figure 11).

A second SDS-PAGE was run over a month after the first measurement. The analysis was repeated because results from pulp treatments were suggesting a decrease in the enzyme activity. The second measurement showed fading of the band at 43 kDa and strengthening of the wide bands between 35 and 43 kDa, and 25 and 27 kDa which proved degrading of the endoglucanase (Figure 11). Additionally, a faded band appeared down of the gel. The reason for the degradation is not yet known but could originate from genes in the synthetic expression strain coding protease that has not been fully deleted. The rest of the enzyme preparation was frozen to slow down further degradation. Despite the partial degradation of *Hi* Cel45A, the preparation was still considered suitable for evaluating pH and temperature optima for the enzyme in pulp modification.

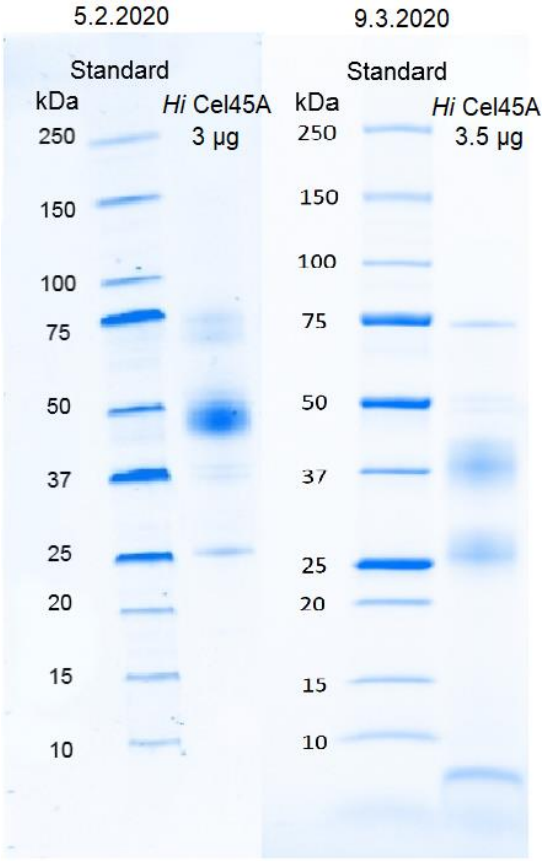


Figure 11. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of VTT produced *Hi*Cel45A endoglucanase after the production (5.2.2020) and over a month later (9.3.2020).

10.3 Temperature and pH optima of *Hi* Cel45A

Optimal pH and temperature conditions for *Hi* Cel45A endoglucanase in pulp treatment were determined at high consistency. The endoglucanase was considered to have the highest activity at pH and temperature values where the viscosity of the pulp samples decreased the most and where the yield loss from the treatment was the highest.

Figure 12 shows intrinsic viscosities and yield losses of the pulp samples after the high consistency enzymatic treatments with varying pH conditions. The results are shown as a function of the average pH of the measured values at the beginning and end of each treatment. All treatments decreased the intrinsic viscosity of the softwood kraft pulp from the initial value of 860 ml/g. Based on the intrinsic viscosity values, *Hi* Cel45A hydrolyses cellulose nearly as effectively at all pH values between 6.3 and 7.7. Nevertheless, intrinsic viscosities of the pulps treated at lower pH values of 5 and 5.6 had decreased less (670 and 650 ml/g respectively) than the viscosities of the samples treated at pH 6.3, 6.6, 7.0, 7.4, and 7.7 (530, 510, 510, 520, and 540 ml/g respectively). At lower pH values, the activity

of the endoglucanase was weaker and on average at pH 5 and 5.6, the reduction of viscosity was only 59 % of the reduction happened between pH 6.3 and 7.7.

In general, the amounts of sugars and oligomers cleaved and dissolved during the treatments were low, only 0.44% of the pulp dry weight at maximum and 0.12% at minimum determined utilizing DNS-reagent. The results of yield loss were mainly in good relation with the viscosity results since treatments that decreased viscosity the most had also the highest yield loss values and vice versa. The only exception was the treatment done at pH 7.7 that resulted in only a modest yield loss of 0.14% while the intrinsic viscosity of the sample had decreased to 540 ml/g. The reason for the divergence is not known and would require performing a parallel treatment to confirm the result. In conclusion, *Hi* Cel45A doesn't have a clear optimum pH value for pulp treatment but it acts nearly as effectively at least in pH area between 6.3 and 7.4.

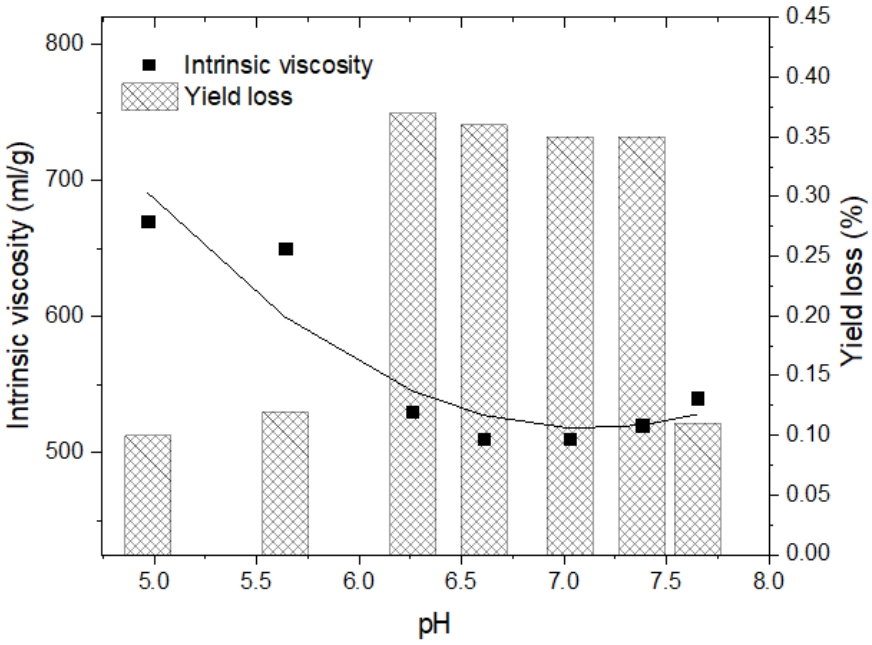


Figure 12. Intrinsic viscosity and yield loss (solubilised sugars) of softwood kraft pulps modified at high consistency (24%) with constant *Hi*Cel45A dose (0.017 mg/g) and varying pH conditions (2h, 50 °C). Initial intrinsic viscosity of the pulp was 860 ml/g.

Temperature optimum of *Hi* Cel45A in pulp treatment was determined by performing a series of five treatments between temperatures 45 and 65 °C. Figure 13 shows intrinsic viscosities and yield losses of the pulp samples after the high consistency enzymatic treatments as a function of the treatment

temperatures. *Hi* Cel45A was found to be equally active throughout a relatively broad temperature range (45–60 °C) whereas at 65 °C the activity started clearly to decline.

Intrinsic viscosities of the pulps treated at 45, 50, 55, 60, and 65 °C decreased the initial intrinsic viscosity from 860 ml/g to 500, 520, 510, 520, and 550 ml/g respectively. Treatment at 65 °C had the smallest impact on the pulp based on the smaller change in viscosity and a modest yield loss. This probably originates from a decrease in endoglucanase stability. However, the decrease in stability during two hours of treatment is not remarkable based on the changes in viscosity values and yield loss results which are only 14 and 38% smaller than the results showing the highest effects respectively.

The amounts of sugars and oligomers dissolved during the treatments were low, only 0.47% of the dry pulp weight at maximum and 0.29% at minimum determined utilizing DNS-reagent. The results of yield loss were in good relation with the viscosity results and did not indicate true differences between the four low-temperature treatments since the yield losses of them were so close to each other (0.36–0.47%).

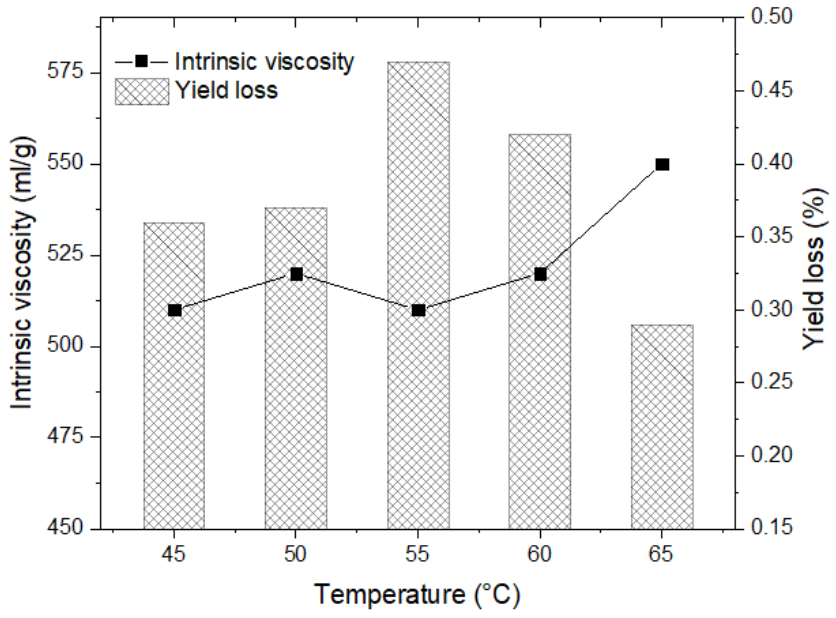


Figure 13. Intrinsic viscosity and yield loss (solubilised sugars) of softwood kraft pulps modified at high consistency (24%) with constant *Hi*Cel45A dose (0.017 mg/g) and varying temperature conditions (2h, pH 6.5). Initial intrinsic viscosity of the pulp was 860 ml/g.

10.4 Combination of endoglucanase and hemicellulases in high and low consistency pulp treatments

Combinations of endoglucanase and hemicellulases in enzymatic treatment of softwood kraft pulp were studied at high (20%) and low (3%) consistencies. The purpose of the treatments was to decrease the DP of pulp cellulose with endoglucanase and lower the hemicellulose quantity with mannanase and xylanase.

Figure 14 presents intrinsic viscosities and yield losses of the samples modified at high consistency. Viscosities of the pulps decreased with increasing enzyme doses. Intrinsic viscosity of the sample treated with only endoglucanase decreased from the initial viscosity of 860 ml/g to 440 ml/g. Interestingly, the reduction in viscosity was higher in the other samples with the addition of hemicellulases. Addition of low dose (0.1 mg/g) of mannanase or xylanase decreased the intrinsic viscosity respectively 30 and 50 ml/g units more (410 and 390 ml/g) while the addition of high doses (0.5 mg/g) decreased the viscosity respectively 40 and 60 ml/g units more (400 and 380 ml/g). Therefore, the combination of endoglucanase and xylanase has a higher impact on the sample viscosity than the combination of endoglucanase and mannanase. Simultaneous addition of both hemicellulases resulted in samples with viscosities of 390 and 360 ml/g respectively for low and high doses. The change in viscosity was 1.2 times higher when high doses of both hemicellulases were used together with endoglucanase instead of using endoglucanase alone. Interestingly, in additional treatments utilizing only a high dose (1 mg/g) of mannanase or xylanase without endoglucanase resulted in pulps with intrinsic viscosities of 840 and 850 ml/g. The utilization of a high dose of hemicellulase had only a minor effect on pulp viscosity while the use of a lower dose of hemicellulase together with endoglucanase led to a clearly lower viscosity than what was achieved with only endoglucanase. The use of hemicellulase in addition to endoglucanase seems to affect viscosity partly due to hemicellulose degradation but also due to a small enhancement in cellulose degradation.

The amounts of sugars and oligomers dissolved during the treatments increased strongly with increasing enzyme doses and were in clear relation with the viscosity results (Figure 14). The yield loss determined utilizing DNS-reagent was only 1.0% for the endoglucanase treated sample and even 6.5% for the sample treated with a combination of endoglucanase and high doses of both hemicellulases.

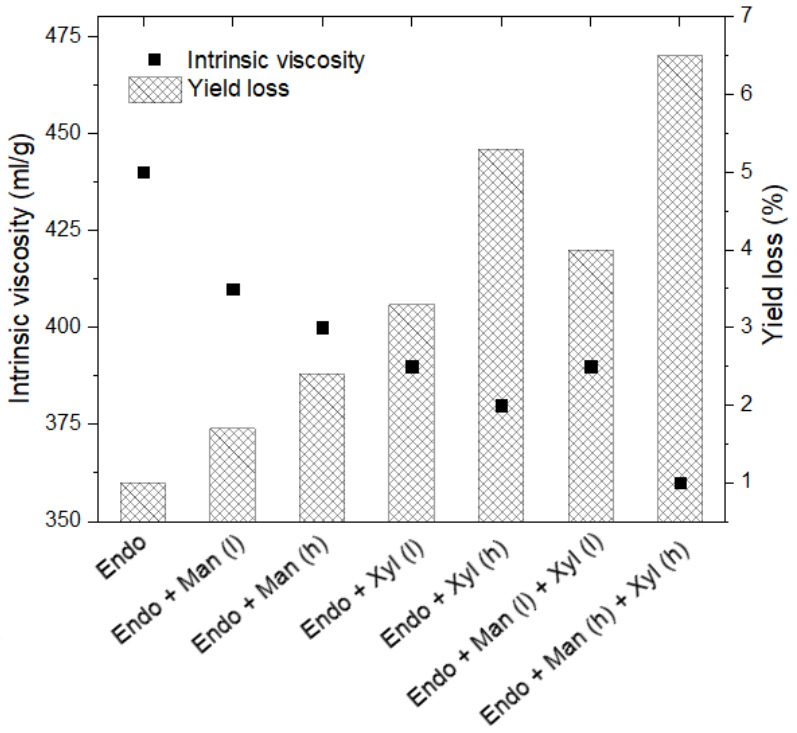


Figure 14. Intrinsic viscosity and yield loss of softwood kraft pulps modified at high (20%) consistency with varying enzyme doses (2h, 50°C, pH 6). The dose of endoglucanase was kept constant (0.02 mg/g) while the amount of two hemicellulases, mannanase, and xylanase, was varied from low (0.1 mg/g) to high (0.5 mg/g). The abbreviations endo, man, and xyl correspond to endoglucanase, mannanase, and xylanase respectively, and characters l and h correspond to low and high enzyme doses. Initial intrinsic viscosity of the pulp was 860 ml/g.

Intrinsic viscosities and yield losses of the samples modified at low consistency are presented in Figure 15. Results from the low consistency treatments show a similar trend of decreasing viscosity and increasing yield loss with increasing enzyme dose but to a lower extent than the high consistency treatments. High consistency treatments decreased intrinsic viscosity 1.9 times more and had 2.1 times higher yield losses on average than the low consistency treatments. The higher degree of modification at high consistency correlates with the earlier discovered enhancing effect of simultaneous enzymatic and mechanical treatment that occurs at high consistency pulp treatment. At high consistency, the enzymes adsorb more on the substrate surface and the fibres induce mechanical friction to each other during the mixing.[56,65]

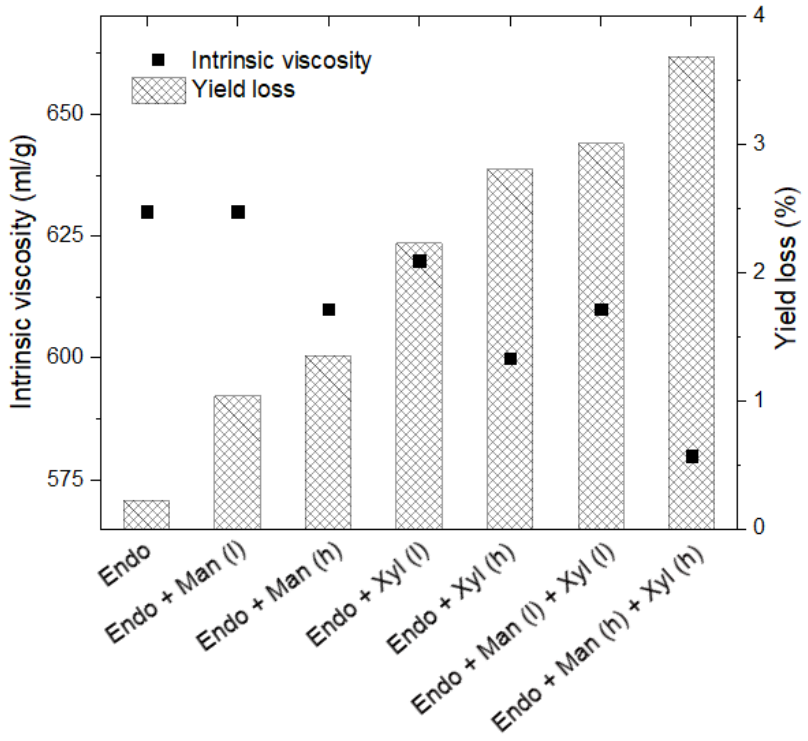


Figure 15. Intrinsic viscosities and molar masses of softwood kraft pulps modified at low consistency (3%) with varying enzyme doses (2h, 50°C, pH 6). The dose of endoglucanase was kept constant (0.02 mg/g) while the amount of two hemicellulases, mannanase, and xylanase, was varied from low (0.1 mg/g) to high (0.5 mg/g). The abbreviations endo, man and, xyl correspond to endoglucanase, mannanase, and xylanase respectively, and characters l and h correspond to low and high enzyme doses. Initial intrinsic viscosity of the pulp was 860 ml/g.

The intrinsic viscosities of the samples treated at low consistency were between 580 and 630 ml/g. The change in viscosity was 1.2 times higher when endoglucanase was used together with high doses of both hemicellulases than if endoglucanase was used alone. This was equally large for the high consistency treatments. The yield losses of the samples from low consistency treatments were only 0.2% at the minimum and 3.7% at maximum. Similarly, as in high consistency treatments, a combination of endoglucanase and xylanase was more efficient than the combination of endoglucanase and mannanase.

The composition of the solubilised sugars (yield losses) from the high and low consistency treatments are presented in Figure 16. The amount of monomeric sugars was determined with high performance anion exchange chromatography after mild acid hydrolysis. The bar chart reveals that the amount of dissolved glucose deriving from cellulose is different between the high and low consistency treatments but remains nearly constant through both of the series. The yield losses of cellulose deriving from the high consistency treatments are 2.7 times higher on average than the ones of low consistency

treatments. When the cellulose yield losses are closely compared, a small increase in cellulose degradation is noticed with the addition of hemicellulases. For the high consistency treated pulps, the yield loss of cellulose was 1.1 times higher when endoglucanase was utilized together with high doses of both hemicellulases than the yield loss from the sample that was modified only with endoglucanase. This suggests that utilizing endoglucanase together with hemicellulases has a minor enhancing effect on endoglucanase action. This enhancement is higher in the low consistency treatments as the addition of high doses of both hemicellulases resulted in 1.3 times higher cellulose yield loss. Earlier in other studies, the removal of hemicellulose has been found to improve enzymatic degradation of cellulose in biomass and hemicellulose has been suggested to act as a barrier to enzymatic hydrolysis of cellulose.[159] Still, the effect of hemicellulose in cellulose degradation is thought to be ambiguous.[159] The effect of hemicellulases enhancing the cellulose degradation is probably smaller among the high consistency treatments because the mechanical action on fibres opens the cell wall structure for the endoglucanase and diminishes the effect of the hemicellulose barrier and the need of hemicellulases.

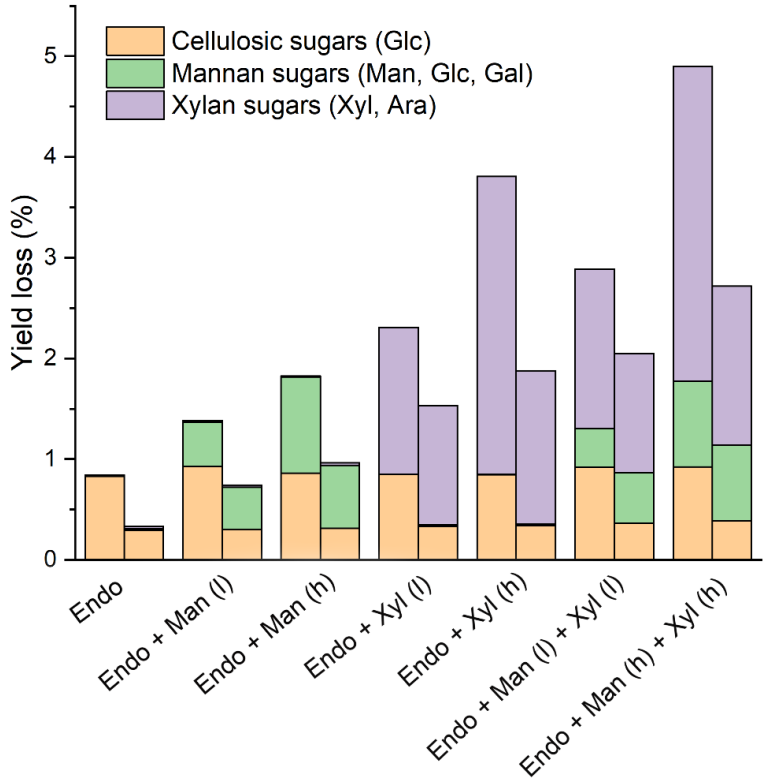


Figure 16. Yield loss (solubilised sugars) deriving from softwood kraft pulp treatments performed at high (20%) and low (3%) consistencies with varying enzyme doses (2h, 50°C, pH 6). The dose of endoglucanase was kept constant (0.02 mg/g) while the amount of mannanase and xylanase were varied from low (0.1 mg/g) to high (0.5 mg/g). The left bar of each section corresponds to high consistency treatment while the right bar corresponds to low consistency treatment. The abbreviations endo, man and, xyl correspond to endoglucanase, mannanase and, xylanase respectively, and characters l and h correspond to low and high enzyme doses.

Similarly as indicated by the other results, xylanase released more sugars and oligomers than mannanase. Most of the hemicelluloses were cleaved at high consistency treatment with the highest enzyme doses. At maximum, 11% of the total galactoglucomannan and 40% of the total arabinoglucuronoxylan were released. The high difference between degradation of galactoglucomannan and arabinoglucuronoxylan probably partly derives from the used pH condition which was not optimal for the used mannanase. The treatment consistency did not have as distinct effect on the yield loss of hemicelluloses as it had for the yield loss of cellulose. The amounts of released sugars from galactoglucomannan and arabinoglucuronoxylan were only 1.1 and 1.6 times higher, respectively, at high consistency treatments while the amount of cleaved glucose from cellulose was 2.7 times higher at high consistency treatments. However, the difference between hemicellulose degradation in high and low consistency treatments was more apparent with higher hemicellulose doses, the yield loss was 1.3 and 2.0 times higher for mannanase and xylanase respectively at high consistency. Part of hemicelluloses is shown to adsorb onto fibres during kraft cooking.[115,160] Therefore, the modest difference in hemicellulose degradation between the high and low consistency treatments with low hemicellulase doses could derive from the amount of accessible hemicellulose. The amount of accessible hemicellulose is probably high enough for the low dose of enzymes even without the mechanical action.

The amount of dissolved sugars from arabinoglucuronoxylan seems to be only dependent on the treatment consistency and the dose of xylanase. However, yield loss of galactoglucomannan at high consistency is 0.8 smaller when xylanase is used in addition to endoglucanase and mannanase than the yield loss resulting from using only endoglucanase and mannanase. In contrary, in low consistency treatment, all three enzymes together cleaved 1.2 more galactoglucomannan than what was cleaved using only endoglucanase and mannanase.

The information on the composition of the yield losses helps to interpret the reasons behind the decreased viscosities. Generally, viscosity of a sample is relative to the cellulose degree of polymerization. This applies well to samples with high cellulose content but is not explicit to describe DP of cellulose in paper grade pulps that still contain a relatively high content of hemicellulose. In this work, the addition of hemicellulases decreased the sample viscosities mostly by enhancing the cellulose degradation but additionally by degrading hemicellulose (Figures 14 and 15). Hydrolysis of branched hemicellulose chains decreases their hydrodynamic volume affecting the overall viscosity of the modified pulp. The increments in cellulose yield losses are low similarly as is the yield loss resulting from the treatment with endoglucanase alone which still led to a strong decrease in the pulp viscosity.

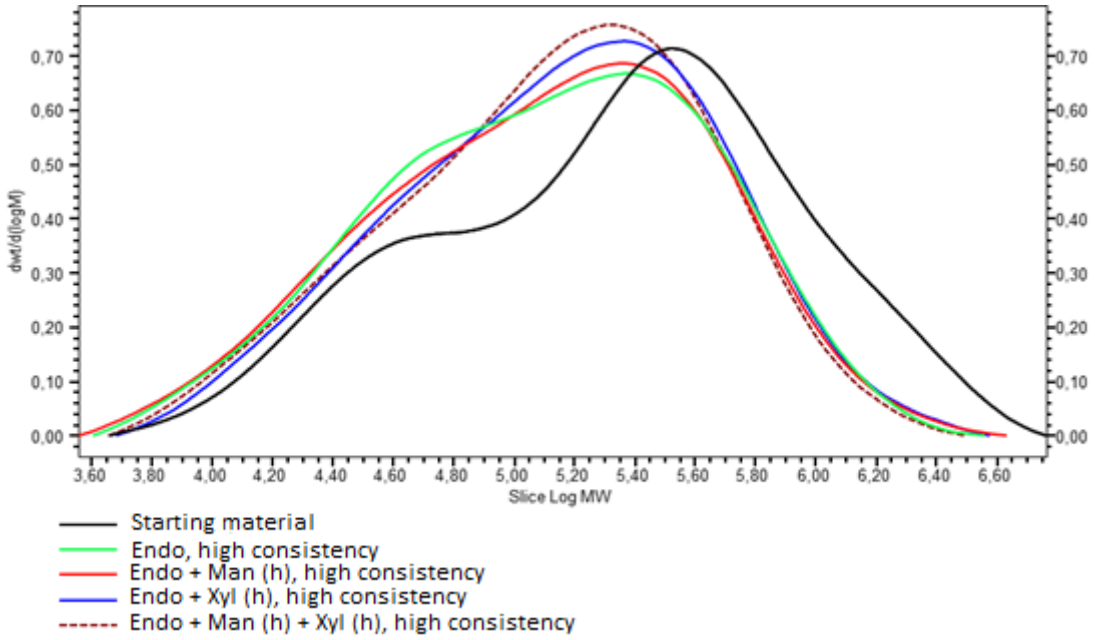
For example, at high consistency endoglucanase alone resulted in cellulose yield loss of 0.83% and decreased the intrinsic viscosity by 420 ml/g units while endoglucanase and high doses of both hemicellulases resulted in 0.92% cellulose yield loss and decreased the intrinsic viscosity by 500 ml/g units.

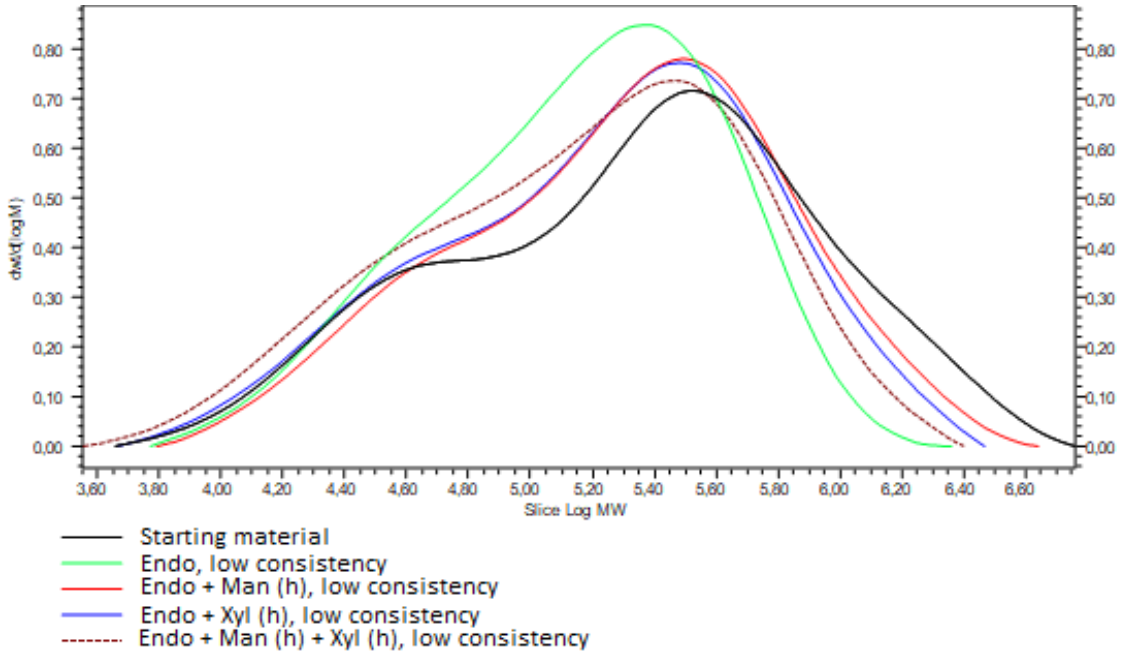
Light microscopy images of the starting material and pulps modified with the highest and the lowest enzyme doses at high and low consistency are presented in the appendix. The images support the viscosity and yield loss results and show a higher level of modification for the pulps treated at high consistency.

10.5 Changes in molar mass distribution

Molar mass distributions were determined from the starting material and the 14 softwood kraft pulps modified at high (20%) and low (3%) consistency with varying enzyme doses. Chromatograms of the starting material and some of the pulps modified at high consistency are presented in Figure 17 and chromatograms of the starting material and some of the pulps modified at low consistency are presented in Figure 18.

The distribution of the starting material distinctly showed one higher peak in the high molar mass area which corresponded to cellulose and a smaller shoulder in the low molar mass area which mostly corresponded to hemicellulose and lignin. All chromatograms of the modified samples displayed a decrease in cellulose DP as the distributions were shifted towards the low molar mass area. Besides, SEC results support the earlier observations of higher cellulose degradation at high consistency treatments because the distributions of the high consistency samples (Figure 17) were farther from the distribution of the starting material than the distributions of the low consistency samples (Figure 18). The high consistency treatments clearly shifted the whole distributions while the low consistency treatments mainly decreased the amount of the longest cellulose chains and only widened the cellulose peak to the side of the smaller molar mass area (Figure 18). In contrary, the shapes of the distributions of the samples treated at high consistency were found to be radically different from one of the starting material. After high consistency treatment (Figure 17), the initial bimodal distribution with two partially separated areas changed to be closer to a unimodal distribution. The smaller peak corresponding to the shoulder of hemicelluloses and lignin in the low molar mass area hid under the overlapping signals from the newly fragmented cellulose chains (Figure 17).





Figures 17 and 18. Molar mass distributions of starting material and softwood kraft pulps modified at high (20%, Figure 17) and at low (3%, Figure 18) consistency with varying enzyme doses (2h, 50°C, pH 6). The dose of endoglucanase was kept constant (0.02 mg/g) while the amount of mannanase and xylanase were varied. The abbreviations endo, man, and xyl correspond to endoglucanase, mannanase, and xylanase respectively and character h corresponds to a high hemicellulase dose of 0.5 mg/g.

The elution times of all of the pulps modified at high consistency were similar meaning that the longest and the shortest chains were nearly equally long between the samples (Figure 17). The similarity

between the plots of the modified pulps supports the observation from the compositional analysis of the yield loss (Figure 16). Because the high molar mass area decreased only slightly with increasing hemicellulase doses, the plots support the assumption that the degradation of cellulose was enhanced only a little by the addition of hemicellulases.

The major differences between the distributions of the pulps modified at high consistency were near the area of the hemicellulose shoulder (Figure 17). The samples treated with sole endoglucanase and endoglucanase together with mannanase had more intense signals in the hemicellulose area (4.5–4.9 log MW) than the other modified pulps. This shoulder was located higher than in the distribution of the starting material because of an increased amount of signals with similar molar masses deriving from the newly fragmented cellulose chains. This small remaining hemicellulose shoulder disappeared completely from the molar mass distributions of the pulps modified with the combination of endoglucanase and xylanase and the combination of endoglucanase and both hemicellulases. This was because xylanase degraded arabinoglucuronoxylan more efficiently than mannanase degraded galactoglucomannan (Figure 16) and decreased the remaining hemicellulose shoulder so strongly that it was left under the signals from the cut cellulose chains.

Molar mass distributions of the pulps modified at low consistency differed from each other more than the distributions of the pulps treated at high consistency (Figure 18). Analysis of solubilized sugars revealed 1.3 times higher cellulose yield loss for the pulp treated at low consistency with the combination of endoglucanase and high doses of both hemicellulases than the cellulose yield loss of the pulp treated with only endoglucanase. The same phenomenon was seen in Figure 18 as the high molar mass area in the distribution decreased with increasing enzyme doses. In low consistency treatments, endoglucanase was possibly not able to hydrolyse long cellulose chains inside the fibers without the help of hemicellulases. Respectively, the distribution of the pulp treated with the highest enzyme dose had intense signals in the tail of small molar mass area exposing the increased amount of short chains.

However, the only exception of the series was the molar mass distribution of the pulp modified with only endoglucanase at low consistency which strangely differed from the other distributions of the modified samples and had the smallest average molar masses. Additionally, this pulp had the highest intrinsic viscosity among the pulps modified at low consistency which indicated that it had the highest average molar masses of the modified pulps. The reason for the deviating distribution is unclear since the area given by the RI detector did not differ from the areas resulting from other samples which would have indicated problems in solubility.

The number and weight average molar masses and polydispersities were calculated based on the molar mass distributions and collected to Table 3. The weight average molar mass of the starting material was clearly higher than the ones of the modified pulps. Polydispersities of the modified pulps were lower than the one of the starting material which can be beneficial in the textile application. Additionally, the weight average molar masses of the pulps modified at high consistency were distinctly lower than the corresponding ones of the samples modified at low consistency except for the two first samples from the low consistency treatments which were strangely low. These values did not correlate with the intrinsic viscosities of the samples and the reason for the low M_w values is not known.

Table 3. The number and weight average molar masses and polydispersities of starting material and pulps modified at high (20%) and low (3%) consistencies with varying enzyme doses (2h, 50°C, pH 6). The values are calculated based on molar mass distributions determined by SEC and corrected pullulan calibration. The dose of endoglucanase was kept constant (0.02 mg/g) while the amount mannanase and xylanase were varied from low (l) (0.1 mg/g) to high (h) (0.5 mg/g). The abbreviations endo, man, and xyl correspond to endoglucanase, mannanase, and xylanase, respectively.

	Sample	**M_n (kDa)**	**M_w (kDa)**	**Polydispersity**
	Starting material	95	461	5.0
20% consistency	Endo	58	252	4.4
	Endo + Man (l)	65	254	3.9
	Endo + Man (h)	63	248	4.0
	Endo + Xyl (l)	72	268	3.8
	Endo + Xyl (h)	67	259	3.8
	Endo + Man (l) + Xyl (l)	64	261	4.1
	Endo + Man (h) + Xyl (h)	59	243	4.1
3% consistency	Endo	79	231	2.9
	Endo + Man (l)	72	270	3.8
	Endo + Man (h)	94	369	3.9
	Endo + Xyl (l)	88	386	4.4
	Endo + Xyl (h)	76	311	4.1
	Endo + Man (l) + Xyl (l)	91	377	4.2
	Endo + Man (h) + Xyl (h)	74	271	3.7

Hydrolysis of cellulose is responsible for the major changes in pulp molar mass distribution since it makes up most of the pulp. The role of short hemicellulose chains is smaller because of their smaller content and shorter chains, especially when calculating weight average molar mass which highlights the high molar mass chains. Based on the viscosity and yield loss results, molar masses should decrease through the series with increasing enzyme doses. However, the M_w values of both treatment series were not in a decreasing order but the values fluctuated through the series (Figure 19). Therefore, a

small increase in the degradation of cellulose and hemicellulose chains was more easily reflected on the pulp viscosity and the molar mass distribution than on the weight average molar mass.

The differences between the molar mass distribution and the average molar masses within the series were rather small. The analysis of the yield loss composition showed only minor increases in cellulose degradation with the increasing enzyme doses. The reason for the occasional increase in the M_w values was most likely deriving as a result of the yield loss. Many of the short cellulose and hemicellulose chains were degraded and removed during the treatment which increased the average molar mass values. Additionally, the solvent exchange procedure might cause sample loss, especially for the shorter chains which could have decreased the sample concentration and changed the sample composition. Absent of the shorter chains could increase the M_w value calculated from the molar mass distribution because the area under the distribution was normalized. At the moment, there is no alternative activation method that works with softwood kraft pulps that would not result in problems with yield loss before sample dissolution.

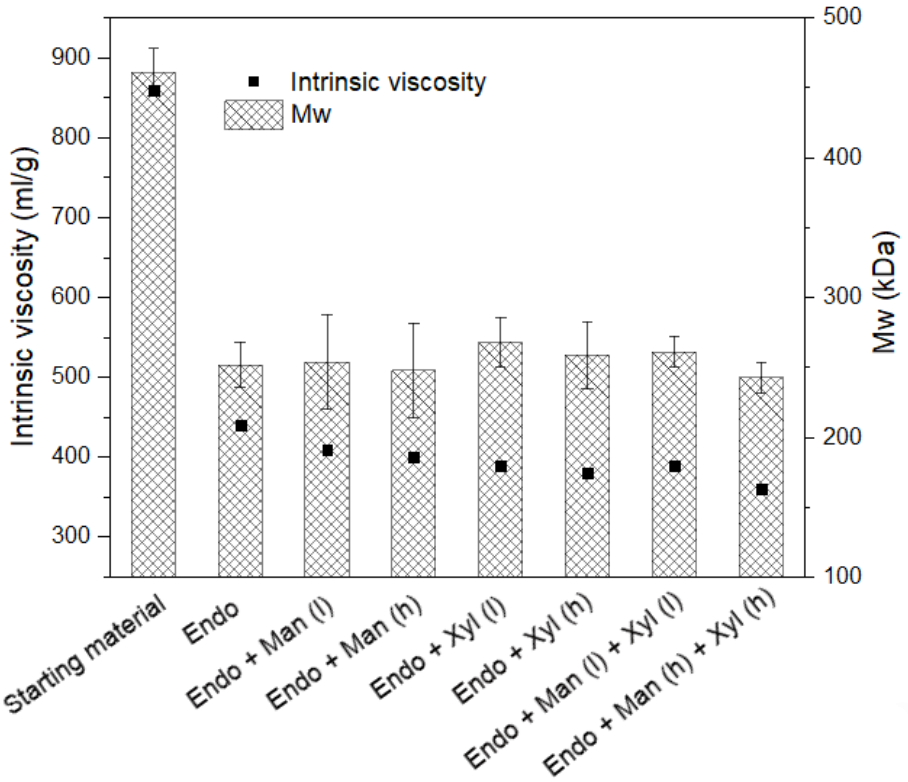


Figure 19. Intrinsic viscosities and molar masses of softwood kraft pulp starting material and pulps modified at high consistency (20%) with varying enzyme doses (2h, 50°C, pH 6). The dose of endoglucanase was kept constant (0.02 mg/g) while the amount of hemicellulases was varied from low (l) (0.1 mg/g) to high (h) (0.5 mg/g). The abbreviations endo, man, and xyl correspond to endoglucanase, mannanase, and xylanase, respectively. The standard deviation of the M_w results is shown in the error bars.

Additional reason for the observed deviation was probably resulting from the challenges related to the dissolution of softwood kraft pulps. Some of the pulps had a major variation in the molar mass distributions between the parallel samples (Figure 20). At least three parallel solutions were prepared from each pulp to be analysed. Molar mass distributions given by the parallel samples were usually similar but four out of 15 pulps had one deviating parallel distribution. These different distributions had fewer signals in the high molar mass area and therefore the calculated average molar masses were lower than the averages of the two other parallel distributions. In addition, the area given by the RI detector, which is related to the amount of sample injected to the column, was significantly lower for the deviating parallel samples. In this case, all the parallel samples should have the same concentration and equally large area from the detector. A smaller area indicated a smaller sample concentration. This together with the lack of high molar mass signals suggested poor dissolution of the samples and especially poor dissolution of the long cellulose chains. The dissolution issues with softwood kraft pulps are commonly known.[114] The sample solution might seem clear during visual inspection but actually, it can contain small insoluble fibrils that are noticed during a more precise investigation such as examing the solution against plane-polarized light.[161]

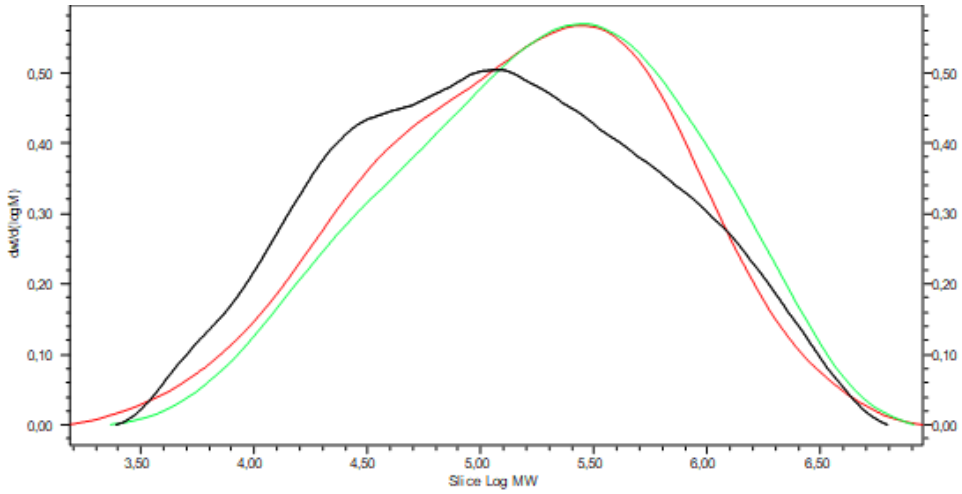


Figure 20. Molar mass distributions of parallel solutions of the same sample, softwood kraft pulp modified with endoglucanase (0.02 mg/g) at high (20%) consistency (2h, 50°C, pH 6). The results are presented against conventional pullulan calibration.

Two additional parallel solutions were prepared from all these pulps with a deviating distribution. All the additional distributions were aligned with the two previous similar distributions. Big deviations

between several simultaneously prepared parallel samples tell about the sensibility of the dissolution method with softwood kraft pulps. Small differences between chromatograms might originate from the heterogeneity of the pulps and the small sample amounts. However, the pulp heterogeneity and small sample amounts might also be one reason for the different solubility between the parallel samples. For example, a sample with high galactoglucomannan content is difficult to dissolve.[114]

10.5.1 Comparison of detection and calibration methods in SEC

Table 4 presents three differently obtained weight average molar masses and viscosity based molar mass for the series of softwood kraft pulps modified enzymatically at high consistency (20%). The weight average molar masses were calculated from the molar mass distributions determined by SEC and detected with MALS and refractive index detector that was either calibrated with conventional pullulan standards or with computational cellulose equivalent molar masses of pullulan standards according to Berggren et al.[89] Viscosity average molar masses were calculated from the intrinsic viscosity values using Mark-Houwink-Sakurada equation (5) where K=0.0101 and a=0.9.[162] Viscosity average molar masses had the smallest values as expected and they decreased through the series according to the intrinsic viscosity values while the weight average molar masses of all the other samples fluctuated during the series.

The weight average molar masses determined utilizing conventional pullulan calibration were the highest of all these values. They were 1.6 and 2.2 times higher on average than the M_w values calculated based on the corrected pullulan calibration and MALS respectively. Values obtained by the corrected calibration were still higher but closer to the results obtained with MALS. The distribution between the values from conventional pullulan calibration was high and varied between 361 and 535 and had a relative standard deviation of 14%. The relative standard deviation decreased to 3% when the calibration was performed with the computational molar masses of pullulan standards. Results from the two different calibration methods varied in the way the values fluctuated through the sample series. For example, the weight average molar masses of pullulan calibrated values of two following samples increased from 408 to 535 kDa while the values of the same samples determined with the corrected calibration decreased from 268 to 259 kDa respectively.

Table 4. Different molar mass values of softwood kraft pulps modified at high consistency (20%) with varying enzyme doses (2h, 50°C, pH 6). The dose of endoglucanase was kept constant (0.02 mg/g) while the amount of hemicellulases was varied from low (l) (0.1 mg/g) to high (h) (0.5 mg/g). The abbreviations endo, man, and xyl correspond to endoglucanase, mannanase, and xylanase respectively. The weight average molar masses were calculated from molar mass distributions obtained by SEC and detected with RI (conventional and computationally corrected pullulan calibration[89]) or MALS detectors. Viscosity average molar masses were calculated using Mark-Houwink-Sakurada equation (K=0.0101, a=0.9).[162]

Sample	**Conventional pullulan calib.** M_w (kDa)	**Corrected pullulan calib.** M_W (kDa)	**MALS** M_W (kDa)	**Based on viscosity** M_V (kDa)
Endo	431	252	176	143
Endo + Man (l)	361	254	181	132
Endo + Man (h)	376	248	231	128
Endo + Xyl (l)	408	268	240	125
Endo + Xyl (h)	535	259	190	121
Endo + Man (l) + Xyl (l)	434	261	178	125
Endo + Man (h) + Xyl (h)	388	243	164	114

The results detected with MALS were not truly reliable and only directional since knowledge of accurate sample concentration was lost during the solvent exchange process and was not possible to solve because calibration constant of RI detector was not known.[112] Additionallt, MALS requires accurate dn/dc value which should be determined separately for samples with varying ratios of cellulose and hemicellulose to obtain high quality results.[76] This was not done due to the time consumption and because of the unknown concentration. Detection of MALS is proportional to the product of molar mass and concentration which results in highlighting the high molar mass. However, additionally MALS was utilized to verify that no aggregation was present in any of the samples.

Overall, the weight average molar masses obtained by the computationally corrected molar masses were closer to the real values than the values obtained with the conventional pullulan calibration. The computational correction should additionally correct the shape of the molar mass distribution and therefore give more actual results. Still, without truly reliable MALS results, it is impossible to conclude which of the calibration methods describes the differences between the pulps best. Additionally, the calibration curves were created with respect to cellulose molar mass which distorts the molar masses obtained for hemicelluloses.

CONCLUSIONS

Series of enzymatic softwood kraft pulp treatments were performed at high (20%) and low (3%) consistency. The purpose was to examine enzymatic pulp treatment as a way to modify paper grade kraft pulp to be a suitable raw material for the future textile industry where ionic liquids could be exploited in cellulose dissolution and creation of wood-based regenerated fibres.

The degree of polymerization of the pulp cellulose was successfully decreased with a relatively small endoglucanase dose. With the combination of endoglucanase (0.02 mg/g), mannanase (0.5 mg/g), and xylanase (0.5 mg/g) the amount of hemicellulose was reduced by removing 11% of the total galactoglucomannan and 40% of the total arabinoglucuronoxylan in high consistency treatment. The utilization of hemicellulases together with endoglucanase was found to have a minor enhancing effect on endoglucanase activity. The high consistency treatments decreased intrinsic viscosity 1.9 times more on average than the low consistency treatments. Dissolution and size exclusion chromatography of softwood kraft pulp was found to be challenging and posses uncertainties related to the accuracy and repeatability of the results.

The high consistency treatments were effectively modifying pulps with low enzyme doses, they were easy to control and reliably repeated. Enzymatic pulp treatment at high consistency seems to be a compatible way to modify paper grade kraft pulp to suitable raw material for textile production. To confirm the suitability of the modified pulps, further studies related to pulp dissolution in ionic liquids, fibre spinning, and fibre regeneration should be concluded.

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Appendix 1. Light microscopy images

Figures 1– 3 present light microscopy images of stained softwood kraft fibres before and after enzymatic hydrolysis performed at high and low consistencies.

Figure 1. Stained softwood kraft fibres used as starting material in enzymatic treatments.

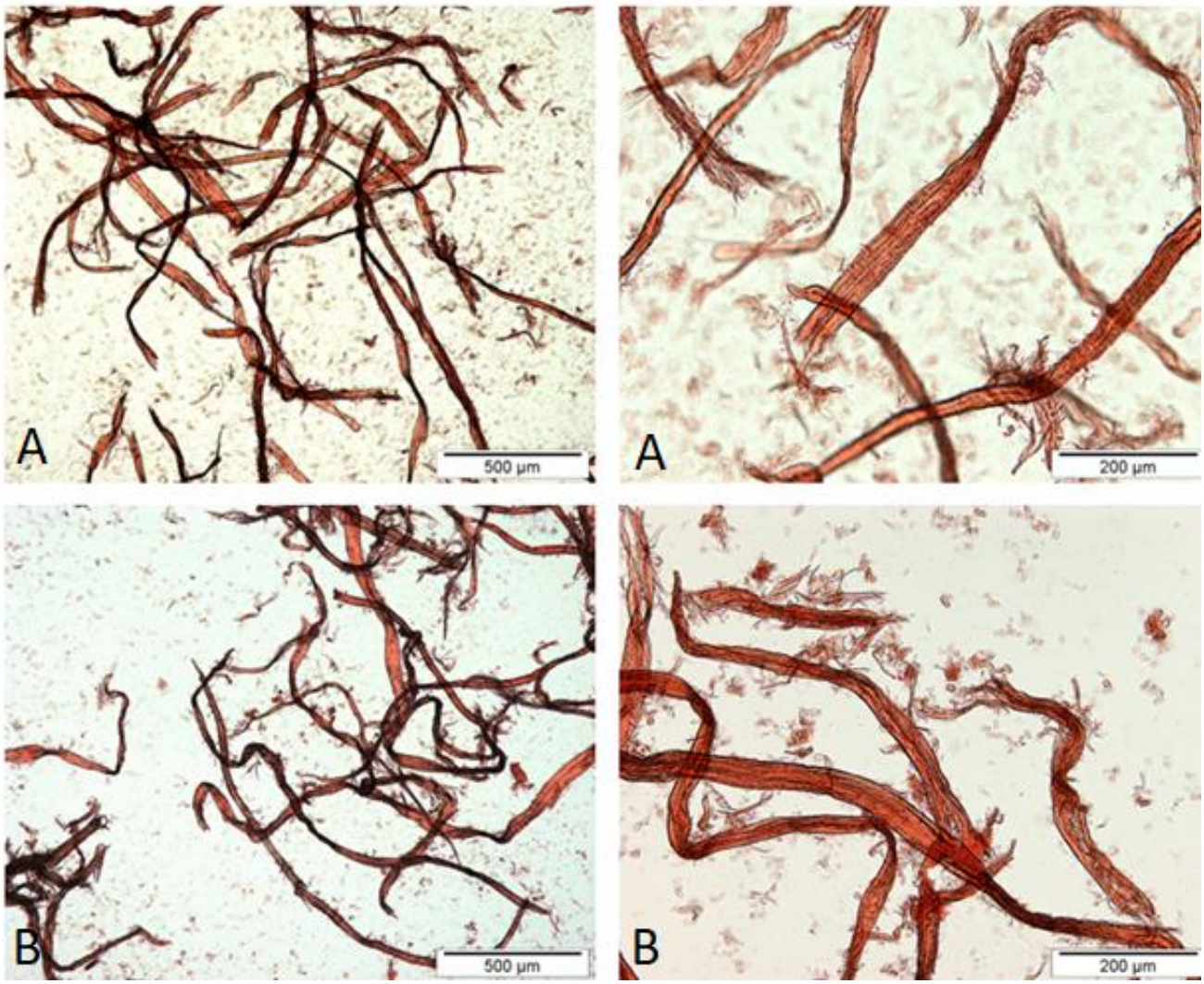

Figure 2. Stained softwood kraft fibres modified enzymatically at high consistency (20%, 2h, 50°C, pH 6) with different enzyme doses. Fibres in images A were modified with endoglucanase (0,02 mg/g) and fibres in images B were modified with a combination of endoglucanase (0,02 mg/g), mannanase (0,5 mg/g) and xylanase (0,5 mg/g).

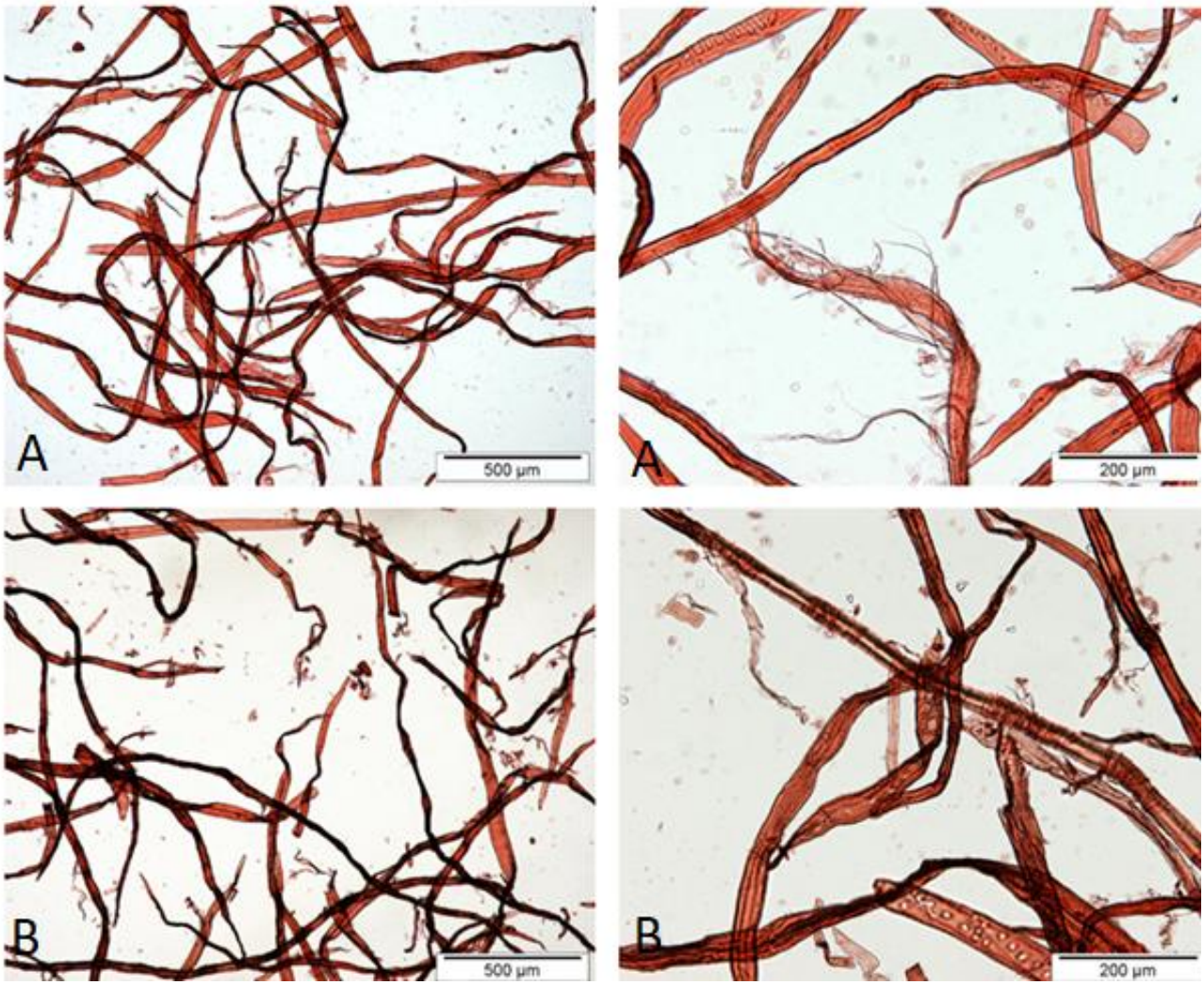

Figure 3. Stained softwood kraft fibres modified enzymatically at low consistency (3%, 2h, 50°C, pH 6) with different enzyme doses. Fibres in images A were modified with endoglucanase (0,02 mg/g) and fibres in images B were modified with a combination of endoglucanase (0,02 mg/g), mannanase (0,5 mg/g) and xylanase (0,5 mg/g).